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## REMARKS

### **Objection to Disclosure**

The objection to the disclosure has been obviated by amendment. Accordingly, withdrawal of this ground of objection is respectfully requested.

### **Claim Objections**

The objection to claim 6 has been obviated by amendment. Accordingly, withdrawal of this ground of objection is respectfully requested.

### **Claim Rejections - 35 U.S.C. § 112, First Paragraph – Written Description**

The written description rejection of claims 1, 2, and 8 under 35 U.S.C. § 112, first paragraph is respectfully traversed. The recitations in the claims of terminology such as “haptten,” “marker group,” “solid phase binding group,” “reactive side groups,” and “predetermined positions” would have been clear to one of ordinary skill in the art based on both the description in the specification and the well-established definitions of these terms.

For example, it is well understood in the art that the term “haptten” refers to a portion of an immunogen. A discussion of the term “haptten” excerpted from *Applications of Fluorescence in Immunoassays* by I. A. Hemmilä (Chapter 2.1.1, pp. 4-7; Tables 8.9-8.12, pages 188-193) is attached herewith as Exhibit A. Moreover, a detailed description of the term “haptten” is also contained in the specification as filed. For example, the specification describes the term “haptten” as including “an immunological reactive molecule having a molecular mass of 100 – 2000 Da” (e.g., page 7, line 33 to page 8, line 1), “immunologically reactive peptide epitopes preferably having a length of up to 30 amino acids” (e.g., page 8, lines 21-22), “nucleic acids with a length of preferably up to 50 nucleotides that are complementary to a nucleic acid sequence which is to be detected in a sample,” (e.g. page 8, lines 30-33), and “peptidic nucleic acids with a length of up to 50 monomeric units” (e.g., page 8, lines 33-35). Furthermore, numerous examples of specific haptens suitable for use in accordance with the claimed invention are provided on page 8 of the specification.

Similarly, it would have been well understood by one of ordinary skill in the art that the phrase "marker group" refers to a detectable moiety or label. A discussion of a related phrase, "luminescence label," excerpted from a review article by A. Mayer and S. Neuenhofer (*Angewandte Chemie international Edition in English*, **1994**, 33, pp. 1044-1072) is attached herewith as Exhibit B (in particular, see pages 1046 ff. and page 1054). A discussion of marker groups is also provided in the *Crockford* reference cited by the Examiner (EP 0155224, page 7). Moreover, a detailed description of the phrase "marker group" may also be found in the specification as filed. For example, the specification describes the phrase "marker group" as including "luminescent metal chelates" (e.g., page 9, lines 3-4) and "fluorescent labels" (e.g., page 9, lines 3-4), numerous specific examples of which are provided on pages 9-11.

In addition, it would have been well understood by one of ordinary skill in the art that the phrase "solid phase binding group" refers to any group through which an attachment can be made to a solid support (e.g., via chemical bond formation, etc). A discussion of solid phase binding groups is provided in the Mayer and Neuenhofer reference cited above (pages 1065 ff.) and in the *Crockford* reference cited by the Examiner (EP 0155224, page 5). Moreover, representative examples of solid phase binding groups suitable for use in accordance with the present invention have been identified in the specification (e.g., page 9, lines 5-8) for purposes of illustration. These include biotin and biotin analogues such as desthiobiotin and iminobiotin.

Furthermore, it would have been well understood by one of ordinary skill in the art that the phrase "reactive side groups" refers to any functional groups (e.g., on the carrier) that can react with functional groups of complementary reactivity (e.g., on the haptens, marker groups, or solid phase binding groups) in order to form a bond. Representative examples of reactive side groups suitable for use in accordance with the present invention have been identified in the specification (e.g., page 9, lines 10-16) for purposes of illustration. These include amino and thiol groups.

Finally, it would have been well understood by one of ordinary skill in the art that the phrase "predetermined positions" refers to locations on the carrier containing a functional group available for reaction with a hapten, marker group, or solid phase binding group, or to the locations of the haptens, marker groups, or solid phase binding

groups themselves. The specific positions will be determined on a case-by-case basis according to the specific requirements of an application. A detailed description of predetermined positions is set forth in the specification (e.g., page 6, lines 5-27).

Inasmuch as all terminology recited in the claims is both described in the specification and would be well understood by those of ordinary skill in the art, Applicants respectfully submit that the claims reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Accordingly, withdrawal of this ground of rejection is respectfully requested.

#### **Claim Rejections - 35 U.S.C. § 112, First Paragraph – Enablement**

The enablement rejection of claims 1- 8 under 35 U.S.C. § 112, first paragraph is respectfully traversed. The specification as filed provides one of ordinary skill in the art the wherewithal to practice the invention commensurate in scope with the present claims.

In accordance with MPEP 2164.08, “[h]ow a teaching is set forth, by specific example or broad terminology, is not important.” *In re Marzocchi*, 439 F.2d 220, 223-224, 169 USPQ 367, 370 (CCPA 1971). While specific examples of polymeric carriers have been set forth in the specification, Applicants also provide direction and guidance of a more general nature to enable one of ordinary skill in the art to make and use alternative polymeric carriers. For example, oligonucleotide carriers and polymeric carriers composed of peptidic nucleic acids are described (e.g., page 7, lines 3-29), and a reference (WO 92/20703) is cited in order to provide additional instruction relating to peptidic nucleic acids and their production (e.g., page 7, lines 21-23).

Similarly, while specific examples of marker groups have been set forth in the specification, Applicants also provide direction and guidance of a more general nature to enable one of ordinary skill in the art to make and use alternative marker groups. For example, numerous luminescent metal chelates other than ruthenium bipyridine are described (e.g., page 9, line 26 to page 11, line 18), together with a description of the effects of charge on the electrochemiluminescence reaction (e.g., page 11, lines 5-18). In addition, several references (EP 178450, EP 255534, EP 580979, and WO 90/05301)

are cited in order to provide additional instruction relating to alternative metal chelates and their production (e.g., page 10, lines 28-34). Furthermore, as alternatives to metal chelates, numerous fluorescent labels are described for use as marker groups in accordance with the present invention.

For this reason and for the reasons set forth above, Applicants respectfully submit that the claimed invention is fully enabled. Accordingly, withdrawal of this ground of rejection is respectfully requested.

#### **Claim Rejections - 35 U.S.C. § 112, Second Paragraph**

The rejection of claims 1-8 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention, is respectfully traversed. As outlined below, each of the phrases identified in paragraphs A-H in section 15 of the Office Action has been described in the specification and/or has a well-defined meaning within the art.

As noted above and in the Mayer and Neuenhofer reference, the phrase "solid phase binding groups" refers to groups that can react specifically with a binding partner on a reactive solid phase. Thus, this phrase describes a specific interaction between a solid phase binding group and a solid phase resin.

As noted above, the phrase "predetermined positions" is determined on a case-by-case basis by one of ordinary skill in the art practicing the claimed invention. The precise locations on the carrier are not restricted, but rather are chosen to coincide with desired points of attachment of haptens, marker groups, or solid phase binding groups to the carrier chain.

The phrase "nucleotide analogues" is well understood in the art, and refers to nucleotides that are structurally similar to other nucleotides apart from one or more structural differences. As used in the claimed invention, the phrase "nucleotide analogues" refers to carriers that retain their capacity to bind haptens, marker groups, and/or solid phase binding groups regardless of any such modifications to their nucleic structure.

As noted above, the phrase "reactive side groups" is well understood in the art and has been explained in the specification (e.g., page 9, lines 10-16). The phrase

refers without limitation to groups suitable for coupling haptens, marker groups, and solid phase binding groups to the carrier chain, and includes moieties such as amino and thiol side groups. As described in the specification, reactive primary amino side groups "can be produced by incorporating appropriate monomers e.g., amino acids such as lysine, ornithine, hydroxylysine or cysteine into the carrier chain" (page 9, lines 14-16).

The recitation of the "coupling of multiple hapten molecules" to a multiplicity of "reactive side groups" refers to individually coupling each hapten molecule to each individual reactive side group. For example, as described in the specification, the process involves "selectively incorporat[ing] additional haptens...at positions of the carrier chains at which monomers are located having free functional groups" (e.g., page 6, lines 9-13). However, this recitation does not require that every hapten-containing monomeric unit in the carrier chain contain the same type of hapten.

The recitation of covalently binding monomeric units to hapten molecules, marker groups or solid phase binding groups "via primary amino groups or thiol groups" has been described in the specification. For example, as noted above, reactive primary amino side groups "can be produced by incorporating appropriate monomers e.g., amino acids such as lysine, ornithine, hydroxylysine or cysteine into the carrier chain" (page 9, lines 14-16). Furthermore, it is described in the specification that thiol side groups can be provided by an amino acid such as cysteine (e.g., page 12, lines 15-16).

The recitation of "protective groups" that are "selectively cleavable" and the recitations of "acid-labile groups" and "acid-stable groups" are well understood in the art. A series of reactivity charts excerpted from *Protective Groups in Organic Synthesis*, 3<sup>rd</sup> Edition by Theodora W. Greene and Peter G. M. Wuts (John Wiley & Sons, Inc., New York, 1999) is attached herewith as Exhibit C. These charts serve as guides to the selective protection and/or selective deprotection of various functional groups. As shown in Reactivity Charts 8-10, numerous amino protecting groups are known, some of which are readily cleaved under acidic conditions (i.e., an "H" or "M" in the columns corresponding to pH < 7), others of which are stable except to alkaline conditions (i.e., an "H" or "M" in the columns corresponding to pH > 7). The concept of "orthogonal protection" (i.e., employing at least two protecting groups each of which can be

selectively cleaved) is also referred to in the *Tam* reference cited by the Examiner (col. 8, lines 9-30). Moreover, a detailed description of selective cleavable protecting groups is also contained in the specification as filed. For example, the specification describes the use of acid-labile amino protecting groups such as Boc, and acid-stable amino protecting groups such as phenyl acetyl (e.g., page 13, line 34 to page 14, line 24).

For at least the reasons set forth above, Applicants respectfully submit that the present claims are not indefinite. Accordingly, withdrawal of this ground of rejection is respectfully requested.

### **Claim Rejections - 35 U.S.C. § 102**

The rejection of claims 1-8 under 35 U.S.C. § 102(b) as being anticipated by *Crockford* (EP 0155224) is respectfully traversed. *Crockford* does not teach or suggest the carriers of the claimed invention.

*Crockford* was cited in the International Search Report as a category "A" reference during the international phase of the present application. Such a categorization is reserved for a "document defining the general state of the art which is not considered to be of particular relevance." In particular, *Crockford* does not teach or suggest "forming a carrier on a solid phase by linking together monomeric units ... selected from the group consisting of nucleotides, nucleotide analogues and amino acids," as called for by the claimed invention. Rather, the description of carrier molecules in *Crockford* is limited to sucrose polymers (e.g., Ficoll<sub>70</sub>) and bovine serum albumin (e.g., page 7, lines 25-26; claims 10, 24, 29).

Inasmuch as *Crockford* fails to teach or suggest the carriers of the claimed invention, Applicants respectfully submit that the claimed invention is neither anticipated by nor would have been obvious in view of this reference. Accordingly, withdrawal of this ground of rejection is respectfully requested.

The rejection of claims 1-4, and 7-8 under 35 U.S.C. § 102(b) as being anticipated by *DeLeys* (WO 93/18054) is respectfully traversed. *DeLeys* does not teach or suggest introducing into carriers "monomeric units covalently bound to hapten

molecules and ... monomeric units covalently bound to marker groups or solid phase binding groups,” as called for by independent claims 1 and 8 (emphasis added), nor does it teach or suggest introducing into carriers “monomeric units comprising reactive side groups” and coupling hapten molecules and marker groups or solid phase binding groups thereto, as called for by independent claim 2.

The peptides described in *DeLeys* are simply (a) biotinylated and (b) bound to a solid phase. There is no teaching or suggestion to introduce into these peptides monomeric units covalently bound to hapten molecules as well as monomeric units covalently bound to marker groups or solid phase binding groups. Indeed, even if the biotin described in *DeLeys* is regarded as a solid phase binding group in the sense of the claimed invention, and the peptides described in *DeLeys* are regarded as carriers in the sense of the claimed invention, then at least one element of the claimed invention—namely, 1-10 monomeric units covalently bound to hapten molecules—would still be lacking.

For at least these reasons, Applicants respectfully submit that the claimed invention is neither anticipated by nor would have been obvious in view of *DeLeys*. Accordingly, withdrawal of this ground of rejection is respectfully requested.

The rejection of claims 1, 3, and 5-8 under 35 U.S.C. § 102(b) as being anticipated by *Tam* (US 5,229,490) is respectfully traversed. *Tam* does not teach or suggest introducing into carriers “monomeric units covalently bound to hapten molecules and ... monomeric units covalently bound to marker groups or solid phase binding groups,” as called for by independent claims 1 and 8 (emphasis added), nor does it teach or suggest introducing into carriers “monomeric units comprising reactive side groups” and coupling hapten molecules and marker groups or solid phase binding groups thereto, as called for by independent claim 2.

The multiple antigen peptide systems described in *Tam* are principally directed to the production of vaccines based on peptide type antigens (e.g., col. 10, lines 27-29). Although *Tam* contains a generic statement that dendritic polymer systems described therein could potentially be labeled with detectable labels (col. 10, lines 44-46), there is no teaching as to how such labels should be attached to the dendritic polymers, nor



indeed at what positions they should be attached. Moreover, *Tam* contains no teaching or suggestion of introducing into a carrier (e.g., a dendritic polymer) monomeric units covalently bound to hapten molecules as well as monomeric units covalently bound to marker groups or solid phase binding groups, as recited in the claimed invention. Indeed, even if the antigens described in *Tam* are regarded as hapten molecules in the sense of the claimed invention, and the dendritic polymers described in *Tam* are regarded as carriers in the sense of the claimed invention, then at least one element of the claimed invention would still be lacking—namely, 1-10 monomeric units introduced into the carrier and covalently bound to marker groups or solid phase binding groups.

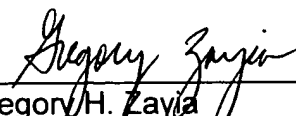
For at least these reasons, Applicants respectfully submit that the claimed invention is neither anticipated by nor would have been obvious in view of *Tam*. Accordingly, withdrawal of this ground of rejection is respectfully requested.

### CONCLUSION

In view of the Amendments and Remarks set forth above, Applicants respectfully submit that the claimed invention is in condition for allowance. Early notification to such effect is earnestly solicited.

If for any reason the Examiner feels that the above Amendments and Remarks do not put the claims in condition to be allowed, and that a discussion would be helpful, it is respectfully requested that the Examiner contact the undersigned agent directly at (312)-321-4257.

Respectfully submitted;

  
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## VERSION WITH MARKINGS TO SHOW CHANGES MADE

Amendments to paragraph beginning on page 5, line 25 and continuing to page 6, line 27:

“When using the conjugates according to the invention that contain 1 – 10 hapten molecules and a defined number of marker or solid phase binding groups as antigens in an immunological method of detection it is surprisingly possible to achieve [a considerable] considerably higher sensitivity and precision and at the same time [at] a reduced lower detection limit compared to known monomeric and multimeric antigens. Moreover the conjugates according to the invention can be constructed in a simple manner by solid phase synthesis e.g., a peptide solid phase synthesis. For [this] these monomeric units, e.g. amino acid derivatives, that are derivatized by a hapten molecule or a marker or solid phase binding group can be incorporated at predetermined positions. In addition it is possible to selectively incorporate additional haptens or marker or solid phase binding groups after completion of the solid phase synthesis at positions of the carrier chain at which monomers are located having free functional groups. This enables a defined and reproducible incorporation of hapten molecules and marker or solid phase binding groups into the conjugate. The distances between individual groups on the conjugate can be exactly defined and varied if necessary. The signal quenching can be kept low by selecting the distance of the marker groups on the conjugate so that the signal strength increases [proportionaly] proportionally to the number of marking groups. A defined spatial orientation of marker groups also contributes to the improvement of the signal strength e.g. in the case of helical carriers. The distances between marker groups are therefore preferably 3-6 or/and 13-16 monomeric units in the case of helical carriers e.g., single-stranded or double-stranded nucleic acids.”

Amendment to claim 6:

6. (Once Amended) The process as claimed in claim 2, wherein the reactive side groups are primary amino groups and the protective groups are selectively cleavable.

**EXHIBIT A**

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# Applications of Fluorescence in Immunoassays

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# PREFACE

Fluorescence provides a diversified and sensitive detection system applied in the versatile field of immunological techniques. The application of antibodies labeled with fluorescent probes dates back to the 1940s, when Coons et al. introduced the microscope immunofluorescence staining technique. During the 1970s fluorescence was considered as a promising and potentially very sensitive detection system in the search for alternative labels to replace radioisotope tracers in immunoassays. Regardless of the number of assays developed and also successfully applied in certain areas, the inherent vulnerability of fluorescence detection to background interferences hindered its application in assays requiring high sensitivity. The recent development of fluorescence instruments, assay technologies and fluorescent probes has, however, resulted in assay techniques producing one of the highest available sensitivities, and fluorometric immunoassays also offer real alternatives to the sensitive radioisotopic immunoassays. The ability of fluorometric detection to combine spectral, temporal and spatial resolution offers a powerful tool for future immunoassay development, too.

The present monograph describes the basic prerequisites for a fluorometric immunoassay; the antibody, the immunological technology, the fluorescent probe and the instrument, as well as gives a profile of the clinical applications of the various assay technologies.

The author would like to express his gratitude to Mrs. Aini Tahvonen for recording the fluorescence spectra included in the monograph, Mr. Reino Harju, M.Sc., for updating the authors knowledge about lasers and detectors, and to Mr. Pertti Hurskainen, M.Sc., for proofreading the chapter discussing DNA-based assays.

ILKKA A. HEMMINKI

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the inherent nitrogen as a marker. The use of fluorescent compounds as sensitive marker substances coupled to antibodies was invented by Albert Coons and his colleagues in the early '40s, when they developed immunofluorescence staining techniques for microbes (5, 6).

The study of antibody production in diabetic patients treated with insulin led to the development of the radioimmunoassay in the late '50s by Berson and Yalow (7, 8); this method has had a major impact on the acceptance of immunological techniques in the field of routine clinical diagnosis.

In their early days radioimmunoassays were exclusively applied for determinations of peptide hormones. Since the pioneering work of Landsteiner in 1946 (9), antibodies have also been produced for small (molecular weight under 10,000) compounds called haptens, for compounds which as such are unable to elicit antibody production but must be bound to larger carrier molecules to form immunogenic conjugates. The production of antisera against haptenic molecules, such as steroids (10) or thyroid hormones (11), opened a new dimension for immunoassays. Since then antibodies have been produced against an enormous number of antigens and biological and synthetic compounds, and these have been applied in a variety of ways for analyzing those compounds. Modern biotechnology has revolutionized antibody production, and genetic engineering opens totally new perspectives for their future applications.

## 2.1. IMMUNOGENIC RESPONSE

An antigen is an immunogenic compound which can elicit a strong immune response in an immunized animal. An immunogenic antigen can be a peptide, protein, polysaccharide, polynucleotide, or almost any polymeric compound containing functional groups on its surface recognized by antibody-producing B-lymphocytes. The primary recognition by the membrane bound receptor proteins of lymphocytes triggers the complex process of maturation of antibody producing B-cells and the subsequent production of large quantities of antibodies.

The production of antisera of high titer, affinity, and specificity requires substantial amounts of chemically pure antigens. A large amount is needed for repeated immunizations of test animals. High purity is an absolute necessity in order to obviate cross-reactivities with unrelated compounds. The purification and stability problems with some biological compounds can be a limiting factor in antiserum production, but these have been partly overcome with the development of methods for producing monoclonal antibodies (Chapter 2.2).

### 2.1.1. Haptenic Antigens

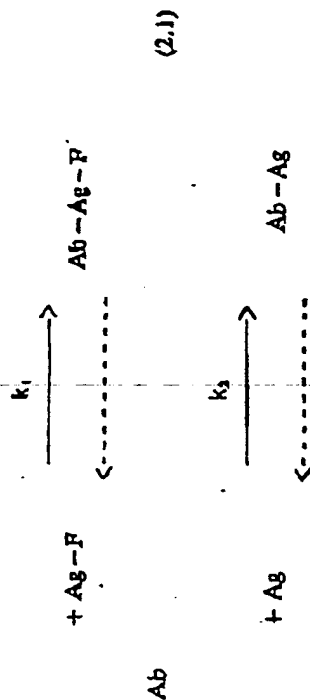
Haptenic antigens are compounds which because of their small size cannot elicit immune response. Generally the molecular weight limit for immunogenic response is around 10,000. Because of the difficulties in producing anti-hapten antibodies, the first real immunoassays were developed for peptides or proteins, and actually the first "specific protein binding assays" of haptenic molecules, developed by Roger Ekins et al. in the early '60s, used naturally occurring specific binding proteins, thyroxine binding globulin for labeled thyroxine (12) and intrinsic factor for labeled B<sub>12</sub>-vitamin (13).

The production of anti-hapten antibodies was invented in the late '40s (9), and anti-steroid antibodies were produced in 1957 (10). It was several years, however, before these were applied for making radioimmunoassays. For eliciting immune response the haptenic molecules need first to be coupled to a suitable carrier. Bovine serum albumin is the most often used carrier protein for immunizations, mainly because of its solubility and availability. Other proteins, like keyhole limpet hemocyanin, have been preferred later on because of their high immunogenicity and coincident contribution of the production of anti-hapten antibodies with high titer and affinity (14).

The production of anti-hapten antibodies of predetermined specificity is often problematic, partly because the coupling of the compound to a carrier can block important epitopic sites needed for specificity and partly because of recognition of the linking arm between the hapten and carrier by the produced antibodies. Since the antibodies are able to bind structures equal to about 7 amino acid residues (15), an anti-hapten antibody most often recognizes simultaneously part of the linking group and spacer arm used in conjugation reaction for immunization (bridge recognition).

Bridge recognition is especially problematic for steroid immunoassays (16, 17) and is encountered when labeled steroids (traces) or immobilized steroids (e.g., solid-phase reagent) are prepared using the same position of the steroid (site homology) or same linking arm (bridge homology) as used for preparing the immunogenic conjugate. With such conjugates the competitive binding between the limited amount of antibody, labeled antigen, and the unknown amount of sample antigen (or standard) favors the reaction between tracer and antibody with poor replacement; the rate constant  $k$ , is much higher than  $k_r$  (Eq. 2.1). The poor replacement results in insufficient slope to the standard curve and low assay sensitivity because the sample antigen is unable to compete with the tracer in binding to antibodies.

Accordingly, the production of immunogenic conjugates for steroid immunization is better performed after selecting different spacer arms or sometimes even different positions for attachment on the steroid structure (Fig. 2.1).



For example, considerably higher sensitivity was obtained in an assay of 17-hydroxyprogesterone when using a bridge heterologous tracer as compared to a respective homologous system (18). Similarly, the equilibrium time required for ligand displacement in an assay of estradiol shortened from 10 h to 1 min when changing from a homologous system to heterologous (19). The requirement of site homology depends greatly on the analyte and antibodies used. In BIA of cortisol Arakawa et al. (20) used cortisol-6 $\alpha$ -hemisuccinate for producing the antigen conjugate for solid-phase immobilization and 3-carboxymethylcholone conjugate for producing the marker-enzyme tracer. On the other hand, in the experiments of Kobayashi et al. (21) and Mikola and Mäkitinen (22), cortisol could be assayed only with a site homologous system. Tietze and Andries (23) tested spacer arms between estradiol and biotin for use in BIA. They found that a reasonably long spacer was an absolute necessity and that the chemical structure of the spacer may also have a major effect on bridge recognition.

## 2.2. MONOCLONAL ANTIBODIES

In 1975 Köhler and Milstein (24) made the first monoclonal antibodies of predetermined specificity by fusing a spleen cell line producing the specific antibodies with a myeloma cell line capable of continuous growth in cell culture. Since then the advent of monoclonal antibodies has had an enormous impact on many fields of biomedical research (25, 26). It was soon realized that the technique would revolutionize the immunology field as well, and it has raised great expectations also in immunotherapy, imaging, and biotechnology.

Monoclonal antibodies are rapidly gaining a dominant position in immunoassays, especially from a commercial point of view, because of their unlimited supply, molecular homogeneity, and defined, unchanged properties. The production and use of monoclonal antibodies has also been

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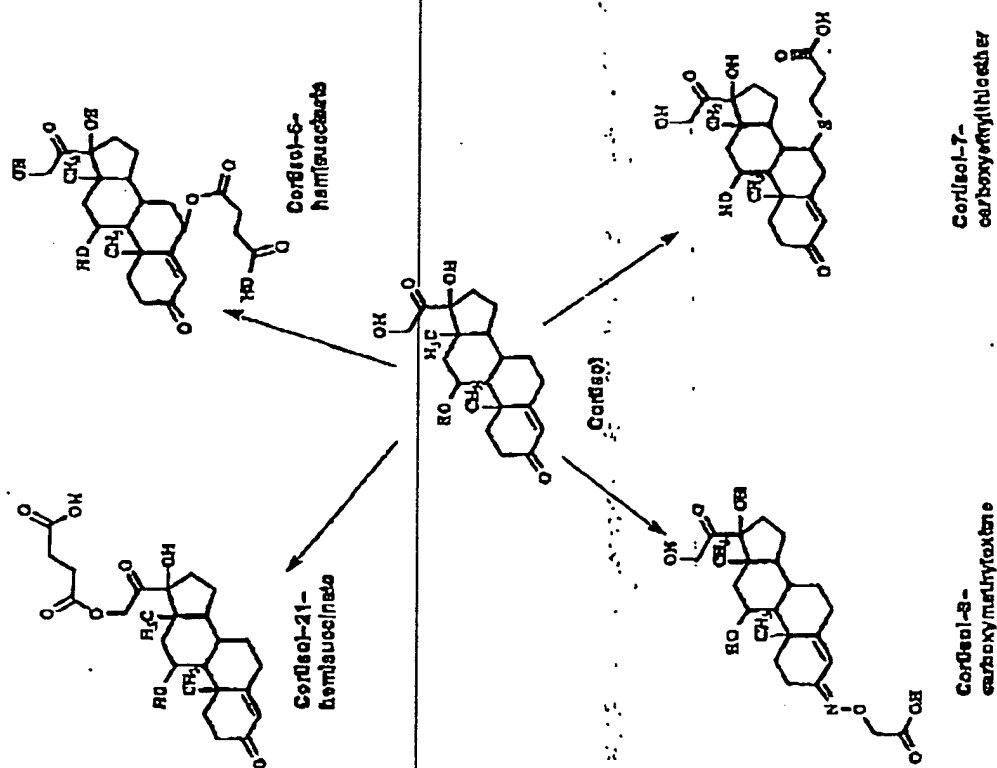


Fig. 2.1. Site heterologous routes to prepare cortisol derivatives for immunization and for labeling.



and it was about 20 years before FPFA reached routine clinical laboratories—when Abbott adopted the technology and developed clinical instruments and numerous kits based on FPFA technology.

Today numerous homogeneous assay principles have been introduced. Several technologies are commercialized and have found quite extensive applications in certain areas, especially in measuring drugs (therapeutic drug monitoring, TDM, and tests for illicit drugs).

### 8.3.1. Fluorescence Polarization Immunassays

The efficiency of light absorption by a fluorophore is dependent on the angle between the electronic dipole of the exciting light and the absorption oscillators of the molecule. A polarized light will excite only those molecules that have their absorption oscillators parallel to the plane of exciting light. The polarization level of the resultant emission depends on the lifetime of the excited state ( $\tau$ ) and the rotational motion of the molecule. For steady-state measurement polarization is generally expressed by the Perrin equation (Eq. 8.1) (1182).

$$(1/p - 1/3) = (1/p_0 - 1/3) (1 + \tau/r_p) \quad (8.1)$$

The rotational relaxation time,  $p$ , can be calculated for a spherical molecule according to Equation 8.2.

$$p = 3 \eta V / kT \quad (8.2)$$

Rotational relaxation time is directly proportional to the volume of the molecule (size and shape) and viscosity ( $\eta$ ) of the medium.

A large molecule, such as an antibody, has a tumbling time typically around 10 to 100 ns, whereas small molecules, such as haptens, have tumbling times around 0.1 to 1 ns. In steady-state polarization measurement (continuous excitation with polarized light), the resulting polarization of emission depends on the size and shape of the labeled substance and the ratio of rotational relaxation time to the decay time of the fluorochrome. This forms the basis for measuring binary binding reactions—for example, in immunoreaction.

To be practical for an immunoassay, the change in molecular volumes during the immunoreaction needs to be high enough, such as it is during the binding of the haptenic tracer to its antibodies. It gives a practical limit for the size of antigen, which should be below 20,000. The decay time of the fluorophore needs to be longer than the rotational time of the haptenic tracer but shorter than the rotational time of the formed complex. Fluorescein ( $\tau$

4.5 ns) accordingly works very well for normal FPFA, the polarization of which increases drastically upon the binding of the fluorescein-labeled hapten to the respective antibodies.

For large antigens, fluorochromes with somewhat longer decay times have been tested. With proteins the intramolecular tumbling becomes problematic, however, when using steady-state measuring (1183). So far no applications have been made using large binding entities (e.g., microbeads) and long  $\tau$  probes.

In addition to the size limitations of FPFA, problems arise also from the low affinity nonspecific binding properties of serum proteins, especially that of albumin, which increases the polarization level nonspecifically. To avoid the albumin effect, a sample dilution jump has been used (1184), or various sample pretreatments are required. In the pretreatment solutions either chaotropic ions, proteolytic enzymes, protein precipitating reagents, or solvents are used. The pretreatment of samples is especially needed for analytes that require a high sensitivity that does not allow for high dilutions.

The principle of fluorescence polarization was developed by Perrin in 1926 (1182). About 30 years later, in 1952, the technology was applied in biological systems by Weber (1185). For monitoring immunoreactions, the fluorescence polarization technique has been used since 1961, since the pioneering work of Dandliker et al. (277), who studied the interaction of fluorescein-labeled penicillin (279), ovalbumin (280), and estrone (1186) with their specific binding proteins or receptors. Dandliker has also written a number of review articles about the principle and applications of fluorescence polarization (282, 1184, 1187).

The experimental studies of FPFA during the 1960s and 1970s were conducted with research fluorimeters equipped with polarization accessories and have resulted in a limited number of clinical applications (Table 8.9), mainly because of the lack of appropriate instruments for routine assays.

Table 8.9. Early Applications of Fluorescence Polarization in Protein Binding Assays

Analyte	Tracer	Assay type	Reference
Anti-penicillin-Ab	FITC-Penicillin	Direct	279
Estrone receptor	FITC-Estrone	Direct	1186
Anti-ovalbumin-Ab	FITC-Ovalbumin	Direct	280
Anti-Conalbumin-Ab	FITC-Conalbumin	Direct	281
Trypsin	FITC-Dactin	Direct	283
Anti-insulin-Ab	FITC-Insulin	Direct	283
hCG	FITC-hCG	Competitive	282, 1188
Purgicides	FITC-Ag. #	Competitive	1189

Table 8.10. Applications of TD<sub>x</sub>

Analyte	Reference	Evaluation
<b>Antibodies</b>		
Genamycin	1192	1193-1196
Tetramycin	1192	
Amikacin	1192	1197
Streptomycin	1198	
Netilmicin		1195, 1199
bopacide		1200
Vancomycin		1201
Aminocyclitol		1202
<b>Anticonvulsants</b>		
Phenytoin	365, 1203	1194, 1204, 1205
Phenobarbital	365, 1203	1194, 1204
Carbamazepine		1204, 1206, 1207
Valproic acid		1204, 1208
<b>Antiarrhythmics</b>		
Quinidine/free		1209, 1210
Hydroquinidine		1211
Lidocaine		1212
Disopyramide/free	1213	
MEQX	1214	
<b>Other drugs</b>		
Theophylline	1215, 1216	1194, 1217, 1218
Digoxin		1194, 1219-1221
Meclizolone	1222	
Benzodiazepine	1223	1224, 1225
Flecainamide		1226
Paracetamol		1227
Meloxicam		1228
Cyclosporine	1229-1231	1232-1243
<b>Misc drugs</b>		
Morphine	1244	
Amphetamine	1245	1246
MHPG	560	
Barbiturate		1247
Opiates		1247
Cocaine (salab.)	1248	1249-1251
<b>Hormones</b>		
Thyroxine (T <sub>4</sub> )	1252	1253, 1254
FT <sub>4</sub>		1253
T <sub>3</sub>	1255	
T <sub>4</sub> uptake	1256	
Cortisol		1257

Table 8.10. Applications of TD<sub>x</sub> (continued)

Analyte	Reference	Evaluation
<b>Hormones</b>		
Eres cortisol/10		1258
Estriol	1259	
OH-Indole acetate	1260	
<b>Proteins and peptides</b>		
Angiotensin	1261	
CRP	1262	1263, 1264
Transferrin		1265

Wider usage of competitive FPIA in clinical routine started during the early 1980s, when Abbott introduced an automated instrument designed for clinical FPIA applications (455, 456, 1190). With instruments of various stages of automation (TD<sub>x</sub>, AD<sub>x</sub> and IM<sub>x</sub>) and over 50 different kits, FPIA has become one of the most used FIA in clinical chemistry (379, 1191). Lately Abbott has been accompanied by other reagent and instrument manufacturers, such as Roche Diagnostics, CANAM Diagnostics, Colony, Sankyo, Junodon of Oregon Inc. (INNOFLUOR FPIA), Polymed Co. and Source Scientific Systems (Focus FPIA fluorimeter).

The reagent pack of TD<sub>x</sub> generally contains a pretreatment solution, and serum, and antigen labeled with a fluorescent derivative. The instrument performs the required dilutions, records the blank value to be subtracted, and measures the final polarization level. The technology is used primarily in TDM and screening for illicit drugs, but it is also used for some hormones and even for a few proteins, such as globulin, transferrin, and CRP. Table 8.10 summarizes examples of the articles describing FPIA applications performed on TD<sub>x</sub>, AD<sub>x</sub>, or the automated IM<sub>x</sub>, including the numerous evaluations of the existing kits and other FPIA applications of the TD<sub>x</sub> instrument. Some of the assays, such as the assay of cyclosporine, have sparked a great number of evaluations, partly collected in the table.

The research group of Prof. Landon has developed FPIAs since 1976 (1266) and has developed analytical applications for the determination of hormones and drugs. They have been able to simplify the technology further by using a one-step, one-reagent method based on antibodies pre-equilibrated with FITC-labeled antigens. By adjusting the respective affinities so that ligand displacement can take place rapidly, this LIDA principle provides an extremely simple and rapid analysis. Assays are performed with various research fluorimeters, including the Perkin Elmer LS 20 Polarization Fluorimeter particularly developed for clinical routine assays (457). FPIA applications performed with homemade reagents are listed in Table 8.11.

Table 8.11. Research Applications of FPIA

Analyte	Label	Reference
<b>TDM</b>		
Gentamicin	FITC	1265
Phenylephrine	FITC	372
Phenytoin	2-Naphthol-sulfonamide	390
Valproic acid	FITC	1267
Paracetamol	FITC	487, 1268
Quinine	FITC	1269
Theophylline	FITC	489
Theophylline	Umbelliferyl	672
Salicylate	FITC	1270
<b>Illicit drugs</b>		
Opiates	FITC	345
Amphetamine	FITC	486, 1271
Methamphetamine	FITC	344, 488
Benzylisopropylamine	FITC	490
Barbiturates	FITC	1272-1274
Valiylisovalerylamine	FITC	1275
Acidobenzamide	FITC	1276
<b>Hormones</b>		
Cortisol	FITC	370, 373, 1277
Biotin	FITC	1278
Neopentol	FITC	1278
Deoxydihydro	FITC	1279
Biotin	Fluorescein	19
Biotin	Lucifer Yellow	688
Tetosterone	Lucifer Yellow	688

New manufacturers producing FPIA kits have recently emerged. The kits are intended to be measured either with the existing Abbott TD<sub>4</sub> system or with the manufacturer's own instrument, such as the Roche FPIA, developed for the company's Cobas Bio and Cobas Fara clinical analyzers. At the moment, alternative products are concentrated in drug monitoring (Table 8.12).

Relatively little effort has been used to develop FPIAs for larger molecules such as proteins. The problems with proteins are related to their large size and their flexibility, as well as the lower sensitivity and more narrow dynamic range obtainable. TD<sub>4</sub> has, however, been applied to some proteins, such as globulins, ferritin, and CRP (Table 8.10) and to analytes that do not require high sensitivities.

Table 8.12. Alternative Commercial FPIA Assays

Analyte	Company	Reference
Cocaine	Roche Diag.	1280
Tobramycin	IBC, Innochem Diag.	1281
Vancomycin	Roche Diag.	1280
Phenylephrine	IBC, Innochem Diag.	1281
Phenylephrine	Roche Diag.	565
Phenylephrine	Roche Diag.	566, 1282
Phenylephrine	IBC, Innochem Diag.	1281
Phenylephrine	Roche Diag.	565
Phenylephrine	IBC, Innochem Diag.	1281
Phenylephrine	Roche Diag.	566
Phenylephrine	Roche Diag.	1280, 1283
Phenylephrine	CANAM	1281
Phenylephrine	IBC, Innochem Diag.	1281
Phenylephrine	Colony	1284
Phenylephrine	Roche Diag.	566, 1282, 1285
Phenylephrine	Roche Diag.	1286
Phenylephrine	Roche Diag.	1287
Phenylephrine	Roche Diag.	566, 1282
Phenylephrine	Roche Diag.	566
Phenylephrine	Roche Diag.	1280

Urios et al. (1188) have made an FPIA for urine hCG using FITC-labeled hCG as the tracer. Reportedly they observed a rather wide dynamic range in the assay—from 0.27 to 64 µg/ml. Yamaguchi et al. (501) were able to measure insulin with a competitive FPIA with a dynamic range from 40 to 600 mU/ml, but only from pure insulin preparations and not from serum samples. A similar insulin FPIA has also been tested by Nishipaluk and McGown (1288), who studied the fluorescence intensity changes, decay-time changes, and polarization changes of FITC-labeled insulin during the immunoreaction. Assays of smaller peptides, such as angiotensin (1261) and neocortisol (454), can be developed more easily.

One approach toward protein FPIAs has been the development of fluorescent probes with longer decay times. Daneyl and umbelliferone derivatives have been tested for FPIA of CK-MB (617). The longer decay-time emitter, dansyl derivative, proved to be too flexible to give an acceptable polarization level. Urios and Clitona used Lucifer Yellow as a label in a direct assay of IgM (1289). The assay was based on a smaller binding unit, the Fab-fragment of a monoclonal antibody, labeled with the fluorophore and used as a direct reagent for the larger antigen, IgM.

**EXHIBIT B**

## Luminescent Labels—More than Just an Alternative to Radioisotopes?

Andreas Mayer\* and Stephan Neuenhofer\*

Chemical, chromatographic, or spectrometric methods are generally unsuitable for the detection of molecules in the nano- and subnanogram region because of their low sensitivity. The radioimmunoassay (RIA) developed by Yalow and Berson in 1959 combined the high sensitivity of radioactively labeled substances with the high specificity of immunological reactions for the first time. In this way it was possible to detect quantitatively the tiniest traces of substances in the presence of an excess of other, in some cases, similar foreign substances without prior enrichment. Immunoassays have

certainly developed to become the most valuable analytical tool of in vitro diagnostics and are today routinely employed for the detection of endogenous and exogenous substances (e.g. hormones, tumor-associated proteins, bacteria, viruses, toxins, drugs, etc.). The many disadvantages of radioactivity such as the required handling licenses, disposal costs, precautions necessary to prevent risks to health, short shelf-life, and limited sensitivity soon led to the search for other nonradioactive labeling methods. Encouraged by the development of light measuring techniques and

the commercial availability of highly sensitive apparatus, radioactive isotopes as labels are today being replaced increasingly by enzymes, fluorophores, or luminophores. Some of the new luminescent labels have, however, not only facilitated replacement of radioisotopes, but also a breakthrough into what has until now been unattainable levels of sensitivity. The following article reviews the methods of luminescent labeling and their applications mainly in the area of immunoassays.

### 1. Introduction

The detection of substances with reagents which bind to the compound to be determined (analyte) is essentially dependent on three conditions if lower detection limits in the pico- to femtomolar region are to be attained and structurally similar substances are not to be measured in addition. First, the detection reagent must have a high affinity for the analyte so that even an analyte present in trace amounts is determined. Second, the binding of the detection reagent to the analyte should be highly specific; this ensures that substances similar to the analyte do not give rise to a deceptively higher concentration of the analyte, or make a time-consuming and labor-intensive prepurification necessary. Third, the reaction product from the analyte and binding reagent must be sensitive to detection, that is, emit a signal which can be quantified exactly by suitable analytical instruments.

The demands for affinity and specificity are ideally fulfilled by antibodies. This involves endogenous glycoproteins, which in organisms of higher life forms, play a decisive role within the immune system by eliminating harmful substances (bacteria, viruses, toxins etc.). In general, affinity constants of antibodies lie between  $10^{10}$  and  $10^{12} \text{ L mol}^{-1}$ . They are capable of recognizing the smallest structural differences at the molecular level, because only then can they distinguish reliably between exogenous and endogenous substances; confusion between the two would have fatal consequences for the host organism.

The first methods used to label detection reagents and thus make them exactly quantifiable employed radioactive isotopes, of which the  $^{125}\text{I}$  isotope, in particular, is still in use today. The advantages of this  $\gamma$  emitter are its small size (minimization of steric interference), its "hard" signal, which as a result is less prone to interference, and its lower detection limit of approximately  $10 \text{ amol}$  ( $1 \text{ amol} = 10^{-18} \text{ mol}$ ).

The combination of antibody/radioactive labeling led to the introduction of radioimmunoassays at the end of the 1950s.<sup>[1]</sup> These have developed into the most important tool of in vitro diagnostics in medicine.<sup>[2]</sup> All conceivable endogenous and exogenous substances in the body fluids (e.g. blood or serum) taken from a patient are routinely determined quantitatively by radioimmunoassays. Of considerable importance for routine applicability is that despite the complex composition of the serum medium under investigation, in general, further purification

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tion steps are not necessary because of the high specificity of the detection reagent "antibody".

Besides the advantages of radioactive labeling there are also, however, a number of disadvantages: The handling of radioactive materials is regulated (official license) and is, thus, limited. The half-life of 60 days for  $^{125}\text{I}$  isotope is too short to guarantee a longer shelf-life for labeled reagents. Limited signal emission during measurement due to the natural half-life prevents the high detection sensitivity required for some applications.

In order to overcome these disadvantages the search for non-radioactive labeling methods (nonisotopic methods) in immunodiagnosics had already begun long ago.<sup>[3]</sup> At the beginning of the 1970s the use of enzymes as labels was described,<sup>[4]</sup> and in the meantime a large number of stable enzyme labels have become available. In combination with chromogenic or luminogenic substrates, detection of the signal is attributed to the measurement of light as absorption or emission. Emitted light instead of radioactive radiation is also employed in luminogenic direct labeling for the quantification of the analyte concentration. Since the number of photons from samples of luminescent-labeled molecules can be higher than the number of radiation quanta emitted from radioisotopes,<sup>[5]</sup> initially fluorescence detection seemed to have a good chance of a wide application in nonradioactive labeling.<sup>[3,6]</sup> For example, detection of a single fluorescent-labeled protein molecule was successful.<sup>[7,8]</sup> However, because of certain disadvantages associated with the first fluorescent label a significant replacement of the radioactive label was not forthcoming. This was only achieved by further development of luminescent labels and luminogenic enzyme substrates.<sup>[9-13]</sup> Luminescent labels not only dispense with having to handle radioactivity but they also allow more precise diagnostic results on account of enhanced sensitivity, and open up new areas of application.

In 1985 the radioimmunoassay (RIA) dominated the German immunoassay market (83% share) with the greatest turnover in the indication fields endocrinology (thyroid gland, fertility) and tumor diagnosis (Fig. 1). The tendency towards the use of meth-

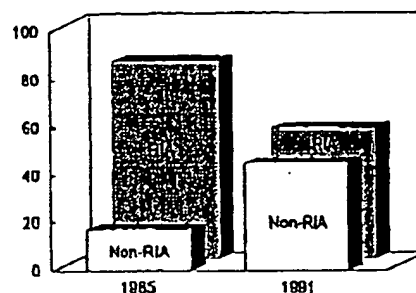


Fig. 1. Market share of radioactive (RIA) and nonradioactive immunoassays (non-RIA).

ods with nonisotopically labeled compounds is shown in the statistics for 1991; the RIA share of the market has sunk considerably to 55%.<sup>[14]</sup> The market share of nonradioactive methods comprises luminescence techniques (e.g. fluorescence immunoassay (FIA), chemiluminescence immunoassay (CIA)), enzyme immunoassay (EIA), and other methods such as nephelometry. A further increase in the market share for methods that work without isotopically labeled compounds is to be expected in the future.

This review article deals in the broadest sense with luminescent labels and their application. After a definition of the term "label" in Section 2 luminescent processes are presented in a simplified energy-level diagram in Section 3. Section 4 describes the enzyme labels in combination with chromogenic and luminogenic substrates. In Section 5 labels for luminescent direct labeling with special consideration of the underlying mechanisms of chemiluminescent labeling are discussed. Section 6 presents several examples of applications in medicine; further important areas of application are in environmental and food analysis.<sup>[15-16]</sup> In addition to the literature already mentioned, the theme of luminescent labels, nonradioactive immunoassays, and gene probes is referred to in several recent books<sup>[17]</sup> and review articles.<sup>[18]</sup>

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A. Mayer



S. Neuenhofer

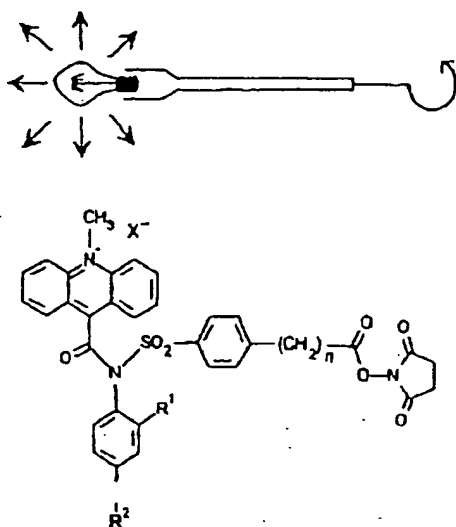
*Stephan Neuenhofer, born 1955 in Mayen/Eifel, studied chemistry and pharmacy in Bonn. After completing his diploma in chemistry (1982) he moved into biochemistry and received his doctorate in 1985 with K. Sandhoff on the topic of gangliosides (Lysogangliosides—Synthesis, Detection in Pathological Brain Tissue and Applications in Biochemical Studies). For his dissertation he received the Edmund-Ter-Meer-Preis. In 1987 he received his approbation as a pharmacist. In August 1987 he began working in the research and development department at the Hoechst AG. His area of interests comprises luminogenic labeling substances, immunoassays, and diagnostic systems as well as their transfer to production. He moved to Behringwerke AG in October 1993 where he has continued with these avenues of research.*

## REVIEWS

### 2. The Label

#### 2.1. Definition and General Structure

A label is a molecule capable of emitting a signal, which is used for labeling proteins and other molecules. It contains, apart from the signal-generating group (fluorescer; more specifically, label), another reactive group (anchor group) which facilitates the covalent bonding to the molecule to be labeled. Between these two groups there is usually a spacer which is supposed to prevent, or at least make difficult, undesirable steric interactions between the signal-generating group of the molecule and the labeled substance. In this way any influences on the immunological reaction ought to be excluded. The schematic structure of a label as well as the chemical formula of a chemiluminescent label from the



Scheme 1. Schematic representation of a luminescent label (top) with the three components: signal-generating unit (fluorescer), spacer, and anchor group as well as a concrete example from the *N*-methylacridinium-9-(*N*-sulfonyl)carboxamide class of compounds (bottom).

class of *N*-methylacridinium-9-(*N*-sulfonyl)carboxamides is shown in Scheme 1. A substance labeled in this way is designated as a tracer.

### 2.2. Luminescent Labels

#### 2.2.1. Definitions of Terms

The term luminescence<sup>(19)</sup> serves as the generic term for most light emission processes such as fluorescence, phosphorescence, chemiluminescence, electroluminescence etc. Exceptions are, for example, glow emission and coherent scattering processes. In practice, three categories are often used, namely luminescence, fluorescence, and phosphorescence. Luminescence serves as the generic term for chemi- and bioluminescence.

#### 2.2.2. Requirements for the Suitability as a Luminescent Label

The suitability of a compound as a luminescent label has certain conditions which must be fulfilled:<sup>(1,20)</sup>

- Coupling to the compound to be analyzed (analyte) must be simple and quite gentle. A large palette of reactive groups is available for this purpose.
- The luminescent properties of the label should not change significantly after the coupling.
- The properties of the labeled substance must not be altered significantly by the labeling. The whole spectrum of characteristics must be taken into consideration, for example physico-chemical properties such as solubility and immunological properties. For the duration of the immunoassay, the immunological reactivity, in particular, must remain sufficiently high.

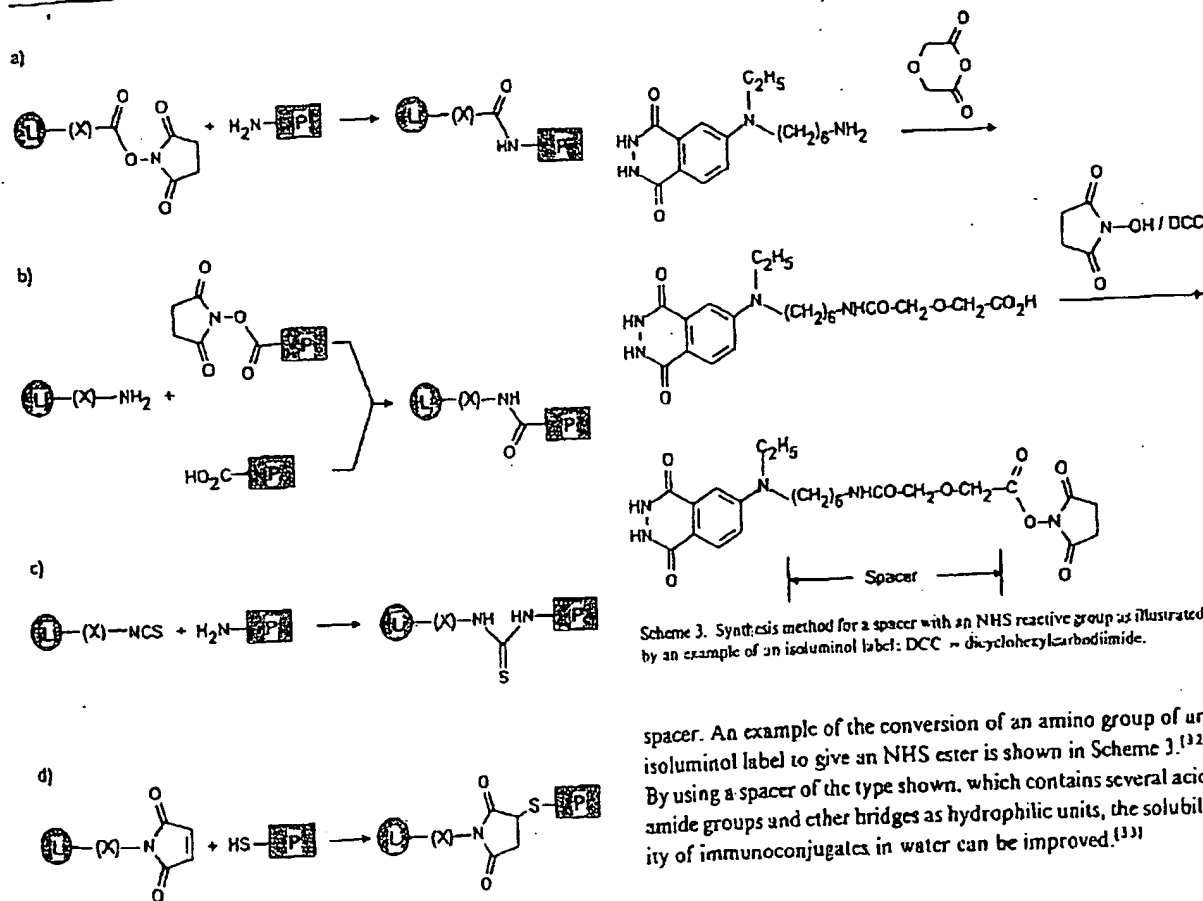
Of course, these general requirements, with the exception of the second point, are also applicable to other labels. The extent to which these points are fulfilled greatly depends on the details of each system and differs from case to case. For instance, small molecules (molecular weight < 2 kD) are altered more significantly than large proteins when labeled with a marker of similar size. Proteins, however, are often more sensitive under the labeling conditions; for example, syntheses cannot be carried out in organic solvents. Nevertheless, for most, the conditions to yield suitable conjugates are established by chemical modification of the signal-generating group and/or of the spacer and by choice of the optimal reactive group.

#### 2.2.3. The Signal-Generating Group

Fundamentally all luminescent compounds can be considered to be signal-generating groups if they exhibit a sufficient quantum yield in aqueous solutions, are stable enough under the conditions employed, and can be functionalized synthetically in such a way that a reactive group can be bound and the properties can be modified, for example, to increase the solubility in water or to change emission characteristics such as wavelength and decay time. Variability is a prerequisite for the broadest possible application of the label. The most important fluorescent and chemiluminescent labels are considered in more detail in Section 5.<sup>(17a, 21)</sup>

#### 2.2.4. The Reactive Group

As mentioned previously, the reactive or anchor group is used to bind the label to the substance to be labeled. Since coupling to biological material such as proteins, antibodies, hormones etc. is frequently necessary, the formation of an acid amide bond between activated carboxyl groups and amino groups is quite common. Many processes for this are known in peptide chemistry which proceed in aqueous solution under mild conditions.<sup>(22, 23)</sup> However, only a few reactions have achieved practical significance for labeling processes. Some of the most important coupling reactions are summarized in Scheme 2. Many luminescent labels have an *N*-hydroxysuccinimide (NHS) ester as the reactive group (Scheme 2a).<sup>(21, 25)</sup> This reactive group has several advantages:<sup>(22, 24)</sup> it can be readily synthesized from carboxylic acid derivatives;<sup>(26)</sup> corresponding labels can be purified to a high degree, for example, by HPLC; thus, labeling can be carried out



Scheme 2. Important labeling processes: L = label, P = protein, X = spacer.

in a defined and reproducible way. With the exclusion of moisture it is possible to store the label over longer periods of time without the reversal of the coupling activity.<sup>[24, 27, 29]</sup> The coupling reaction of the succinimide with amino groups proceeds under mild conditions (room temperature) in aqueous solutions; in contrast, alcohols do not react with NHS esters under these conditions.<sup>[27]</sup> As a variation of this reaction the label can also contain a primary amino functionality as the reactive group for coupling with an NHS ester functionality on the protein (Scheme 2b). The conversion can also be achieved with free carboxyl groups, for example, of proteins, following carbodiimide or "mixed anhydride" methods.<sup>[22, 23, 25]</sup> Recently acridinium ester labels with imido ester reactive groups were described.<sup>[29]</sup> Particularly in the case of fluorescent labels, in addition to the methods already mentioned the isothiocyanate group is often used for coupling with amino groups of proteins to form thiourea derivatives (Scheme 2c).<sup>[30]</sup> The method shown in Scheme 2d for the coupling by thiol addition to maleimide groups is well known in peptide chemistry. This reaction is also employed in luminescent labeling<sup>[21]</sup> and plays a particularly important role in the coupling of enzyme labels to proteins.<sup>[31]</sup>

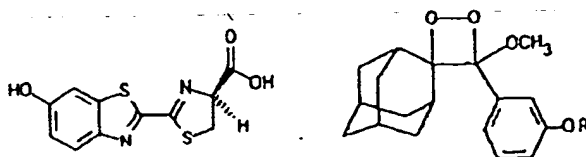
### 2.2.5. The Spacer

Usually simple, short alkyl chains or groups which contain aromatic and aliphatic groups (cf. Scheme 1) are employed as

spacer. An example of the conversion of an amino group of an isoluminol label to give an NHS ester is shown in Scheme 3.<sup>[32]</sup> By using a spacer of the type shown, which contains several acid amide groups and ether bridges as hydrophilic units, the solubility of immunoconjugates in water can be improved.<sup>[33]</sup>

### 2.3. Enzyme Labels

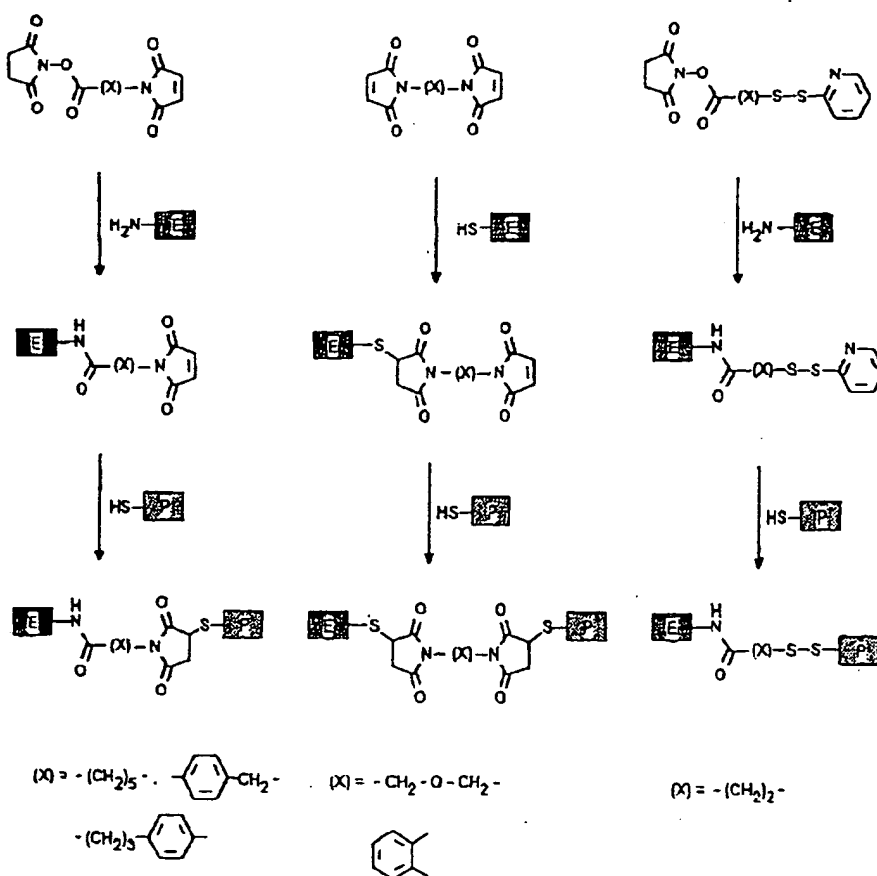
As a result of their practical significance chromogens and luminogens are also dealt with in this article: these are employed as substrates for the enzyme label (Section 4). The reaction of luciferin derivatives (naturally occurring bioluminescent compounds) with their respective luciferase (enzyme which catalyzes the bioluminescent reaction) leads to luminescent reactions with the highest known quantum yields. The leader in this field is the luciferin (Scheme 4)/luciferase system of the North American



Scheme 4. Firefly luciferin (left) and a stable dioxetane derivative (right).

firefly (*Photinus Pyralis*) with quantum yields around 0.9 Einstein mol<sup>-1</sup>.<sup>[11, 15]</sup> Since the end of the 1980s important biogenic luciferases have been genetically engineered and are thus now considered for routine applications.<sup>[15]</sup> High quantum yields are also obtained by enzyme labels with stable dioxetanes (Scheme 4) as luminogenic substrates. The enzymes often employed are alkaline phosphatase and  $\beta$ -galactosidase (the residue R is phosphate and  $\beta$ -galactose, respectively). Such enzymatic systems are dealt with in more detail in Section 4.





Scheme 5. Bifunctional coupling reagents for labeling of enzymes; E = enzyme, P = protein (e.g. antibody), X = spacer.

Since, in general, enzyme labels are not obtainable as stable universally applicable labels, several methods for enzyme labeling should be addressed. The coupling of enzymes to antibodies or fragments of antibodies is often achieved by bifunctional coupling reagents. Some examples are shown in Scheme 5. The reactions are analogous to the methods already mentioned in Section 2.2.4, and details can be obtained from the references cited in the literature.<sup>[24,31]</sup> Coupling methods that exploit the strong noncovalent bonding of the biotin/(strept-)avidin system can only be referred to here.<sup>[24]</sup>

### 3. Electromagnetic Radiation as the Measured Signal

#### 3.1. Comparison of Light and Radioactive Radiation

A considerable disadvantage in the use of radioactive isotopes is the necessity to undertake extensive safety precautions against high-energy  $\beta$ - or  $\gamma$  radiation (up to  $10^{13}$  kJ mol<sup>-1</sup>). Since luminescent labels emit light which is not dangerous—mostly in the visible region of the electromagnetic spectrum ( $E \approx 200$  kJ mol<sup>-1</sup>)—safety measures for the protection from high-energy radiation are no longer required. Furthermore, the specific activity that can be achieved with radioisotopes has an upper limit set by the radiolytic decomposition of the labeled material. Labeling with the isotope <sup>125</sup>I is usually limited to one atom per molecule.<sup>[21,32]</sup> In addition, the half-life of the ra-

dioisotope, which, for example, is only 59.7 days for the frequently used <sup>125</sup>I, limits the storability of the labeled material and detection limits. Moreover, radioisotopes emit radiation continuously even when a signal is not necessary for measurement. For the actual measurement, which normally lasts about a minute, only a tiny fraction of the available signal, can be used. An advantage, however, is that a repeat measurement is possible at any time. Luminescent labels, which have a considerably longer lifetime, emit all the available light within a very short space of time once the light reaction has been triggered. In addition, the activity of the tracer can be enhanced further mainly by multiple labelings; in this way the sensitivity of detection is increased. Repeat measurements on the same sample are, however, not possible, at least in the application of chemiluminescent labels with rapid light emission.

#### 3.2. Photophysical Processes

In the simplified energy-level diagram in Figure 2 the most important photophysical processes are summarized with their typical lifetimes  $\tau$  [s].<sup>[16]</sup> The radiative transitions shown can be used for the production of detection signals. Since radiationless deactivation leads to less efficiency, especially in long-lived phosphorescence processes in solution, phosphorescence detection plays a minor role for luminescent labels. Finally, the quantification in enzyme systems with chromogenic substrates (cf. Sec-

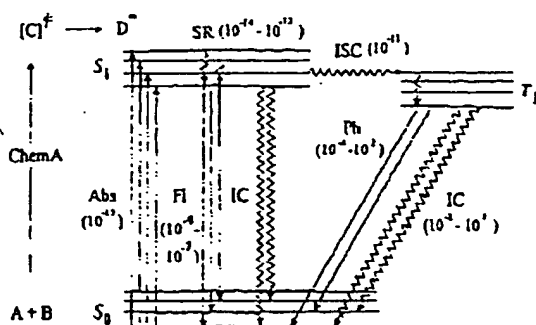


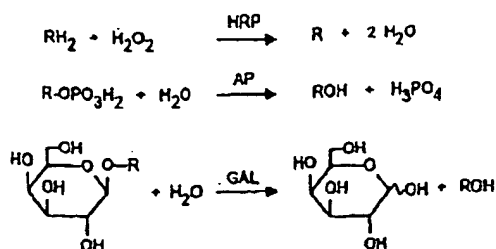
Fig. 2. Simplified energy-level diagram with some photophysical processes. Lifetime  $\tau$  (s) is given in parentheses; Abs = absorption, Fl = fluorescence, Ph = phosphorescence, SR = vibrational relaxation, ChemA = chemical excitation, IC = internal conversion, ISC = intersystem crossing; straight lines represent radiative processes and wavy lines radiationless processes.

tion 4) is ascribable to absorption measurements. Photoexcitation and evaluation of the fluorescence provide the basis for fluorescent labeling (cf. Section 5.1). The production of excited singlet states ( $S_1$ ) by chemical reactions is necessary for chemiluminescence detection. The difference in energy between the  $S_1$  and  $S_0$  states for emissions in the visible region lies between 167 (red light) and 293 kJ mol<sup>-1</sup> (violet light).<sup>(11, 37)</sup> For the effective use of the principles mentioned, in each case, sufficient quantum yields are also a prerequisite. Further details are given for each individual luminophore (see Section 5).

#### 4. Enzyme Labels

The use of enzymes as labels presented the first alternative to radioactive labeling.<sup>(38)</sup> The basic idea is very promising, because no signal-generating compounds are used, but molecules (enzymes) which produce a lot of signal-generating species. In this way an effective signal amplification mechanism is built in right from the outset.

The three most important enzymes that are used as labels are horseradish peroxidase (HRP), alkaline phosphatase (AP), and  $\beta$ -D-galactosidase (GAL). The reactions catalyzed by these enzymes are summarized in Scheme 6. HRP is the smallest enzyme

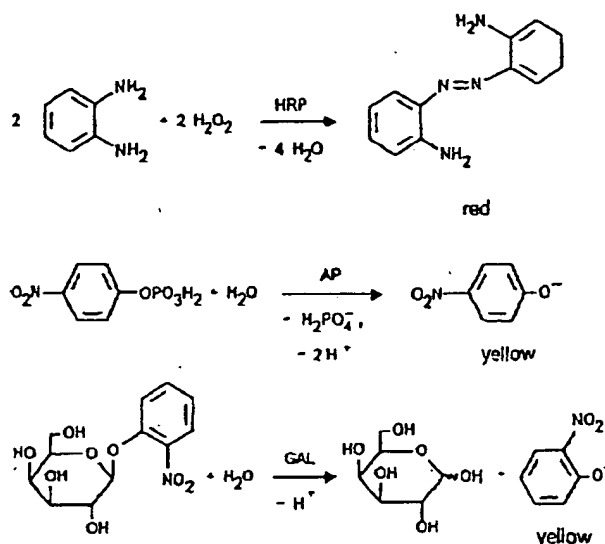


Scheme 6. General representation of the reactions catalyzed by the three most important enzyme labels HRP, AP, and GAL.

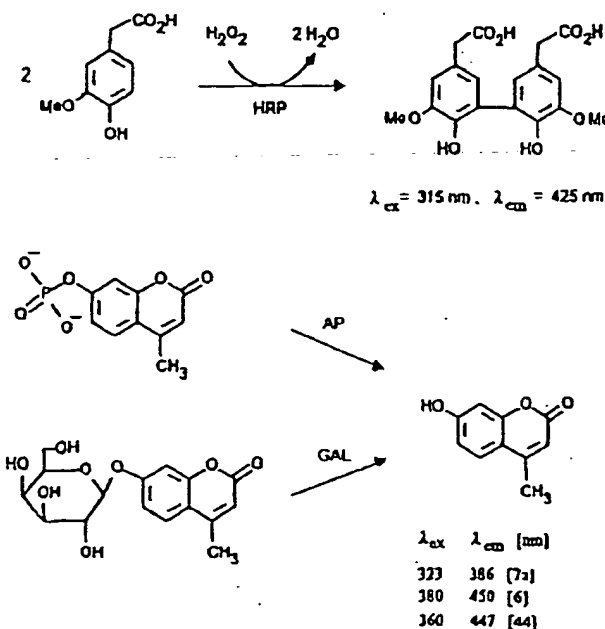
of the three with a molecular weight of approximately 40 kD (AP approx. 100 kD, GAL approx. 500 kD) and as a result presents the fewest steric problems. HRP is, however, sensitive towards antimicrobial agents (azide, Thiomersal) frequently

employed. AP has the highest catalytic activity; however, it is inhibited by phosphate (product inhibition) and ethylenediaminetetraacetic acid (edta; chelates  $\text{Zn}^{2+}$  and  $\text{Mg}^{2+}$  ions which are necessary for enzyme activity).

The oldest enzyme substrates to be employed in analytical methods are the chromogens. These are colorless and are only transformed into colored products by an enzymatic reaction. These products can be quantified photometrically; Scheme 7 shows examples.



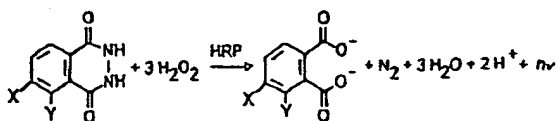
Scheme 7. Examples of chromogenic substrates. Apart from *o*-phenylenediamine, 3,3',5,5'-tetramethylbenzidine (TMB) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) are often employed. Instead of 4-nitrophenylphosphate, 1-naphthylphosphate and 5-bromo-4-chloro-3-indolylphosphate are often employed. In addition to 2- or 4-nitrophenyl- $\beta$ -D-galactopyranoside, chlorophenol red- $\beta$ -galactopyranoside and 5-bromo-4-chloro-3-indolyl- $\beta$ -galactopyranoside are often employed.



Scheme 8. Examples of fluorogenic substrates.  $\lambda_{\text{ex}}$  = excitation wavelength,  $\lambda_{\text{em}}$  = emission wavelength.

The most important fluorogenic substrates of the peroxidases contain the same structural element<sup>[39, 40, 43]</sup>  $p\text{-HO}-\text{C}_6\text{H}_4-\text{C}-$ , whereas in the case of AP and GAL,<sup>[41-43]</sup> 4-methylumbellifer-yl compounds are preferred (Scheme 8).

The chemiluminescent arylhydrazides luminol and isoluminol<sup>[45]</sup> (Scheme 9) are known substrates for HRP. The quantum



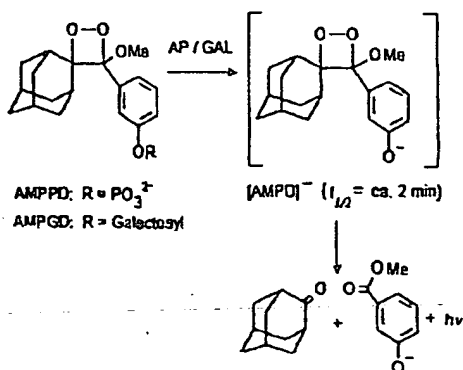
Luminol:  $X = \text{H}, Y = \text{NH}_2$

Isoluminol:  $X = \text{NH}_2, Y = \text{H}$

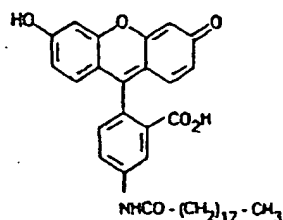
Scheme 9. HRP-catalyzed oxidation of luminol and isoluminol.

yields of the oxidation of these arylhydrazides, that is, the percentage of the molecules of the starting material which at the end of the reaction afford a photon-emitting final product, is approximately 1%. The light intensity can be drastically increased (up to a factor of 1000!) by so-called enhancers such as 6-hydroxybenzothiazole derivatives or *para*-substituted phenols in comparison to a non-enhanced reaction.<sup>[46]</sup> The underlying mechanism of this enhanced chemiluminescence is not completely understood. The most probable explanation is that one or more of the oxidation steps to generate luminol radicals during the complex reaction pathway of the enzymatic oxidation is accelerated.<sup>[46b]</sup>

Dioxetane derivatives are the most important chemilumino-genic substrates for AP and GAL<sup>[47]</sup> (Scheme 10). Also in the



Scheme 10. Adamantylmethoxy(phosphoryloxyphenyl)dioxetane (AMPPD) and adamantylmethoxy(galactopyranosyloxyphenyl)dioxetane (AMPGD) as enzyme substrates. This reaction is catalyzed by AP and GAL.

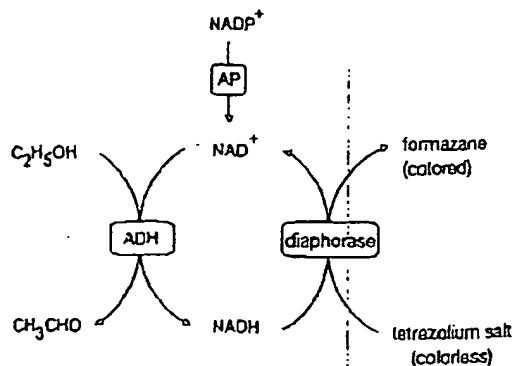


Scheme 11 Example [5-(11-tetradecan-oylamino (fluorescein))] of a fluorescent tenside.

reactions catalyzed by AP and GAL, the chemiluminescence can be enhanced: for this purpose fluorescent tensides are particularly effective (Scheme 11). Together with normal tenside molecules (e.g. cetyltrimethylammonium bromide) they form

micelles into which the substrate diffuses immediately after enzymatic cleavage of the phosphate or galactoside residue. Decomposition of the dioxetane derivative in the micelle leads to an effective transfer of energy to the fluorophore groups of the tenside molecules. This results in a considerable increase in the quantum yield<sup>[47b]</sup> ( $\Phi_{\text{CL}}$  = approx. 0.005 Einstein per mol; for comparison:  $\Phi_{\text{CL}}$  in tenside-free aqueous buffer solution is ca.  $10^{-5}$  Einstein per mol). According to the literature, as few as 600 different enzyme molecules have so far been detected by employing dioxetane derivatives.<sup>[47c]</sup>

In the detection systems described previously the signal-generating group forms immediately in the reaction catalyzed by the enzyme label. There is also, however, a series of detection systems in which the signal-generating group is formed only in a subsequent reaction. In analogy to enzyme-catalyzed reactions, considerable increases in the sensitivity can often be achieved by such coupling reactions. In the detection system of Self,<sup>[48a]</sup> the enzyme label AP catalyzes the formation of  $\text{NAD}^+$  from  $\text{NADP}^+$ . The  $\text{NAD}^+$  formed catalyzes a specific redox cycle from which a colored substance is produced (Scheme 12).



Scheme 12. Example of a signal amplification: The  $\text{NAD}^+$  formed from AP acts as a catalyst in a subsequent redox cycle. ADH = alcohol dehydrogenase.

The lower detection limit for the enzyme label AP with this method was given as 0.01 amol<sup>[48b]</sup> and in a more recent publication<sup>[48c]</sup> even 0.6 zmol (1 zeptomol =  $10^{-21}$  mol); values which would have been inconceivable for direct formation of a dye.

Another method for the highly sensitive detection of AP was described by Christopoulos and Diamandis.<sup>[49]</sup> AP catalyzes formation of 5-fluorosalicic acid from 5-fluorosalicic phosphate. In a subsequent reaction 5-fluorosalicic acid forms a strongly fluorescing ternary complex with  $\text{Tb}^{3+}$  and edta the concentration of which can be quantified by time-resolved fluorescence measurements (cf. Section 5.1.4). The lower detection limit was quoted as being 0.6 amol AP per 50  $\mu\text{L}$  sample volume. In the detection system of A. Bareil et al.<sup>[50]</sup> xanthine oxidase is used as the enzyme label. In the presence of oxygen, it oxidizes hypoxanthine to xanthine and uric acid with formation of superoxide radical anions ( $\text{O}_2^{\cdot-}$ ). Additional reactive oxygen species ( $\text{H}_2\text{O}_2$ ,  $\text{O}_2$ ,  $\text{OH}^\cdot$ ) that form in subsequent reactions are suitable for the chemiluminescent oxidation of luminol.

## 5. Luminescent Labels

### 5.1. Fluorescent Labeling

Fluorescent labels have been used for a wide range of applications in biology, biomedicine, and analytic methods for a long time.<sup>[10]</sup> Applications worthy of mention are fluorescence detection with HPLC after precolumn or postcolumn derivatization,<sup>[11]</sup> flow cytometry,<sup>[12]</sup> fluorescence microscopy,<sup>[13]</sup> DNA analysis,<sup>[14]</sup> and the use as labels in immunoassays, which will be covered in more detail here (see Section 5.1.1). Labeling is usually achieved by the formation of a covalent bond between label—as described, in general, in Section 2—and target substance. Fluorescent dyes without a reactive group can be employed for some purposes. They are only bonded associatively and as a result can accumulate, for example, in cells. A review of fluorochromes that are applied in medicine and biology as well as their spectral data can be found in reference [36].

#### 5.1.1. Special Requirements of Fluorescent Labels for Immunoassays

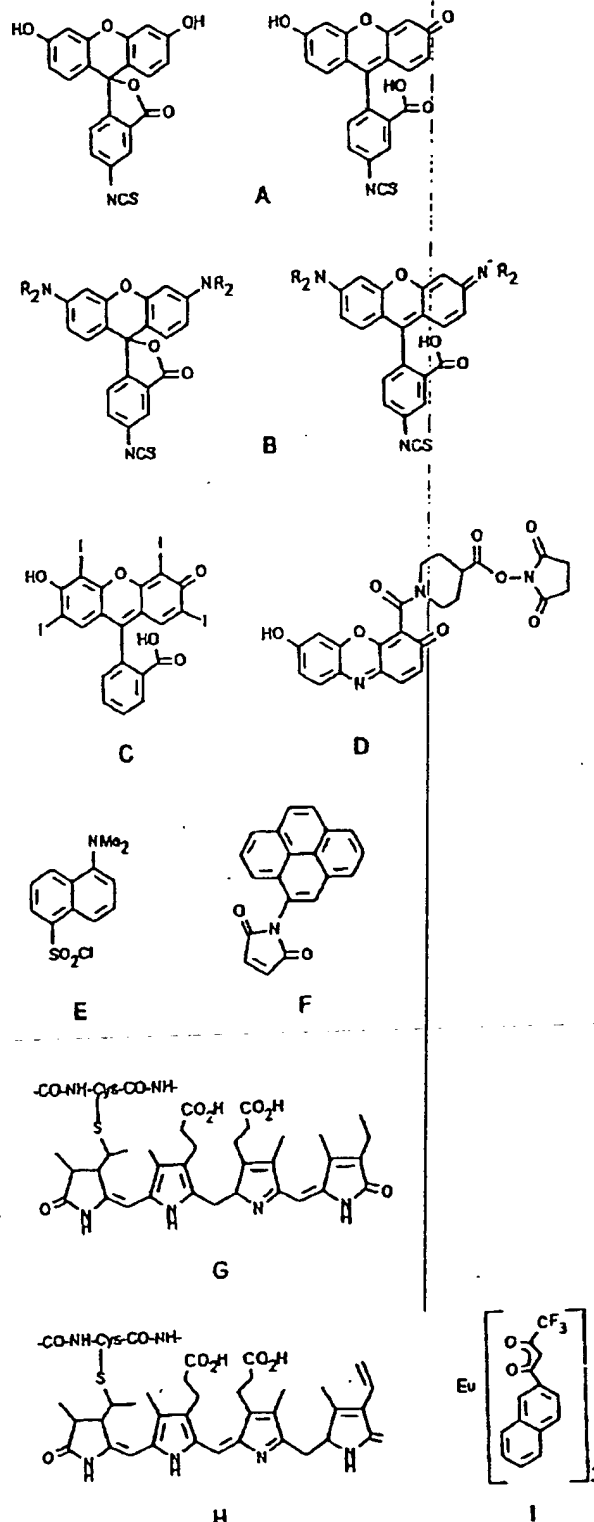
Besides the previously mentioned general requirements for luminescent labels, fluorescent labels for the use in immunoassays should fulfill a few additional conditions which can be derived directly from measured data. In principle, fluorescence measurements of the highest sensitivity are indeed possible,<sup>[6, 7, 55]</sup> but, in practice, the sensitivity of fluorescent immunoassays (FIAs) is, however, drastically limited by background fluorescence, light scattering and quenching effects. The intrinsic fluorescence of serum components is mainly responsible for the background signal<sup>[6, 7]</sup> which covers a broad wavelength region. Serum proteins are excited, for example, at 280 nm and emit at 320–350 nm. Other components such as NADH and bilirubin are excited between 330 and 360 nm and 450 and 460 nm, respectively, and fluoresce in the range 430–470 nm and 515 nm, respectively.<sup>[74]</sup> The detection limit for immunoconjugates of a fluorescent label with bovine serum albumin or immunoglobulin G (IgG) is on average 10 to 50 times worse in serum than in buffer solution.<sup>[64]</sup> Many solid phase materials, for example polystyrene, likewise yield a blank reading. Light scattering is a problem, particularly, in solutions which contain proteins or colloidal dispersed substances. In addition to Rayleigh and Tyndall scattering at the same frequency as the excitation beam, Raman scattering also occurs with a frequency usually shifted by approximately 50 nm. Fluorescence quenching can often result from the smallest changes in the environment of the fluorophore (pH, polarity, oxidation level, proximity of heavy atoms or other absorbing groups). If, for example, a protein is multiply labeled, two fluorophores can become so close that self-quenching of the signal takes place if the absorption and emission spectra overlap.

In order to minimize the influences mentioned the following properties of fluorescent labels are desirable:<sup>[50]</sup> a) longest possible wavelength emission (500–700 nm), b) large Stoke's shift of > 50 nm, c) long fluorescence lifetime of  $\tau > 20$  ns.

A sufficiently long lifetime is particularly significant in applying the principle of fluorescence polarization transfer (cf. Section 5.1.3). Fluorescence lifetimes  $\tau > 100$  ns facilitate a significant improvement of the signal-to-noise ratio and thus of the sensitivity, since measurement can only take place after decay of background fluorescence and light scattering. This principle is applied in time-resolved fluorescence measurements which are explained in more detail in Section 5.1.4.

#### 5.1.2. Labels for Direct Fluorescent Labeling

The first compound used for fluorescent labeling of biological material by Coons et al. in 1941 was anthracene isocyanate for the labeling of bacterial proteins.<sup>[57]</sup> The same group introduced fluorescein isothiocyanate (FITC, A, Scheme 13) as a more ef-



Scheme 13. Selected fluorescent labels A–I. See Table I.

## REVIEWS

fective label soon afterwards.<sup>[58]</sup> Although judging from its spectral data this xanthene dye (cf. Table I) does not completely fulfil the above-mentioned requirements for fluorescent labels,

Table I. Spectral data of selected fluorescent labels A-I.

	$\lambda_{\text{excitation}}$ [nm]	$\lambda_{\text{em}}$ [nm]	$\epsilon$ [L mol <sup>-1</sup> cm <sup>-1</sup> ]	$\tau$ [ns]	$\phi$
fluorescein isothiocyanate A	492	520	$7 \times 10^4$	4.5	0.85
rhodamine B isothiocyanate B (R = Et)	550	585	[a]	3.0	0.7
TM-rhodamine isothiocyanate B (R = Me)	550	580	$5 \times 10^4$	2.0	
erythrosine C	530	558 n [b] 690 ph [b]	$1 \times 10^5$	$2.7 \times 10^{-2}$	0.002
resorufin derivatives D	575	590			
dansylchloride E	340	480-520	$3.4 \times 10^4$	14.0	0.3
pyrene maleimide F	340	375, 392	$3.8 \times 10^4$	100	
phycocyanobilin, protein bound G	614	643	$5.8 \times 10^5$	2.2	0.52 [c]
phycoerythrobilin, protein bound H	546	576	$2.4 \times 10^5$	3.2	0.59 [c]
eurociam-iris[2-naphthoyl-trifluoroacetone] I	340	590, 613	$3.6 \times 10^4$	$7 \times 10^3$	0.6

[a] Unclear literature values: 12 300 [6a,b]; 10 300 [7a]. b)  $\phi$  = fluorescence; ph = phosphorescence. c) Most phycobiliproteins afford  $\phi$  values up to 0.98.

FITC has become the fluorochrome of choice in most applications.<sup>[5, 10]</sup> Despite the large number of fluorescent labels which have been developed since, FITC still remains the most commonly used in fluorescence immunoassays, possibly due to a high quantum yield and stability. Similar properties are exhibited by rhodamines B which belong to the same class of dyes (Scheme 13). Both dyes can exist in the two isomeric forms shown, a spirolactone and quinoid structure.

Efforts to obtain fluorescent labels that can be excited in the longwave region and also emit revealed, for example, that derivatives of the phenoxazin dye resorufin were successful to a certain extent. In the synthesis of fluorescent labels, suitably functionalized resorufins are obtained, for example, from nitroresorcin and 2,6-dihydroxybenzoic acid after reduction of the initially formed resazurine (resorufin-*N*-oxide). Apart from the derivative D shown in Scheme 13 which has a succinimidoyl ester as the reactive group, labels based on resorufin with other reactive groups are also known. Compared to fluorescein, resorufin is less affected by the background fluorescence of serum.<sup>[59]</sup> A longwave shift of excitation and emission wavelengths is also possible with phycobiliproteins,<sup>[60]</sup> which are obtained from different kinds of red and green algae. The structures of the two prosthetic groups are given in Scheme 13 (G, H). The compounds exhibit very high molar extinction coefficients and high quantum yields (> 0.8).<sup>[61]</sup> Not all phycobiliproteins couple with the protein at the A-ring. The substances, which have, in the meantime, become commercially available, were first employed in fluorescence microscopy and flow cytometry, thereafter as labels in immunoassays. When, for example, fluorescein was replaced by phycoerythrin in a sand-

wich immunoassay, a significant increase in sensitivity (factor 2-10) was achieved; however, this was below that expected from the spectral data.<sup>[61, 62]</sup> The size of the label and the difficulty in coupling are unfavorable. Since the phycobiliproteins show a broad excitation and emission band, a parallel determination of several parameters is conceivable by the use of different labels with non-overlapping emission bands. This was confirmed in preliminary experiments.

## 5.1.3. Fluorescence Polarization

The principle of fluorescence polarization,<sup>[63]</sup> known for a long time, was first employed in the antigen-antibody reaction in 1961.<sup>[64]</sup> Fluorescence polarization immunoassay is based on the following principle: if a fluorescing compound in solution is excited by polarized light, the observed emission is also polarized. The degree of this polarization depends upon the rotation relaxation time and, thus, on the size of the molecule. If a small ( $M$ : 1-10 kD) fluorescent-labeled fast-rotating molecule is bound to an antibody ( $M \approx 160$  kD), the result is an increase in the rotating relaxation time of the slowly tumbling immunocomplex and thus also the polarization of the fluorescence. With this principle one can differentiate between unbound labeled antigen and immunocomplex. The method is, however, not suitable for large antigens, since the rotation hardly changes on formation of the immunocomplex. A more exact derivation of the measurement principles is given in reference [63]. The fluorophores mentioned already can be used as labels, although substances with longer fluorescence lifetimes would be more advantageous. In most cases fluorescein isothiocyanate A is employed. Accordingly, the sensitivity is limited by factors mentioned already.

Since no separation step is necessary with this principle (homogeneous immunoassay), determinations can be carried out relatively easily provided that sensitivity in the picomolar region is not required. Fluorescence polarization immunoassay is widely used particularly in the area of drug analysis.<sup>[59, 63]</sup> This method can also be employed in environmental analysis, for example, in the determination of polychlorinated biphenyls (PCBs). Fluorescein derivatives are used as labels.<sup>[65]</sup>

## 5.1.4. Time-Resolved Fluorescence

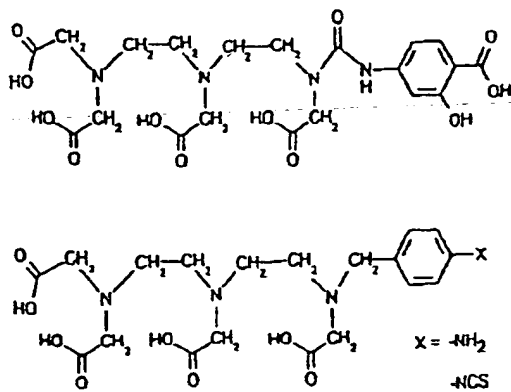
It has already been mentioned that the limiting background fluorescence and the scattering effects can be excluded by the use of labels with very long fluorescence lifetimes and by taking measurements only when the background signal is no longer present. Generally, the lifetime of the unspecific background signal is less than 10 ns. For an interference-free measurement of a specific signal, labels are required whose lifetimes are at least 10 times the decay time of the background.<sup>[66]</sup> Suitable organic fluorophores with lifetimes  $\tau > 50$  ns are, however, very rare. Pyrene derivatives such as F in Scheme 13 exhibit lifetimes of approximately 100 ns, which, however, were shown to be insufficient.<sup>[66]</sup> The lifetimes of phosphorescence processes are considerably longer. The principle applicability of phosphorescent labels, such as erythrosin derivatives in oxygen-free solutions, could indeed be shown<sup>[67]</sup> but no progress with phosphores-

cence was achieved due to its low quantum yields and high expenditure.

It was not until complexes with rare-earth metal ions, primarily europium(III), were employed as labels that prospects of attaining a drastic improvement in the sensitivity of fluorescence immunoassays by time-resolved measurements and developing more sensitive fluorescent alternatives to RIA were realized.<sup>[6, 66]</sup> The use of europium trisdiketonates was proposed by Wiedner<sup>[76]</sup> in 1978 and developed further by other groups.<sup>[68]</sup>

The chelate complexes of europium(III), terbium(III), samarium(III), and dysprosium(III) are distinguished by unique fluorescence properties (cf. I in Scheme 13 and Table 1). Apart from extremely long lifetimes of about 1  $\mu$ s to 1 ms, a very large Stoke's shift (>200 nm) and sharp emission lines impart the complexes with a high sensitivity ( $10^{-14}$  mol L<sup>-1</sup>).<sup>[6, 66]</sup> The reason for the observed lifetimes lies in the excitation/emission mechanism. After excitation of the ligand to the  $S_1$  state and intersystem crossing to an energetically suitable triplet state of the ligand, an effective energy transfer to the resonance state of the metal ion occurs, which then gives rise to a sharp emission characteristic of metal ions.<sup>[6, 66, 69]</sup>

The fluorescent properties of chelates of rare-earth metals alone still do not produce an efficient label for immunoassays. What is essential, also in aqueous buffer solution, is a stable binding to antigens and antibodies. Due to their high stability and solubility in water, polyaminopolycarboxylate chelates, chiefly derivatives of ethylenediaminetetraacetic acid (edta) or diethylenetriaminepentaacetic acid are used for most applications. The use of diazo- and isothiocyanatophenylethylenediaminetriacetate for coordination to europium(III) and terbium(III) has been described.<sup>[66, 70]</sup> Likewise, mixtures of ethylenediaminetriacetic acid, terbium(III), and 5-sulfosalicylic acid,<sup>[70, 71]</sup> mixtures of an edta derivative, europium(III), and a  $\beta$ -diketone<sup>[70, 72]</sup> as well as diethylenetriaminetetraacetic acid derivatives with different trivalent lanthanides<sup>[70, 66, 70, 73]</sup> were employed for the labeling (Scheme 14).



Scheme 14. Ligands for the coordination of lanthanide ions.

Since many of the very stable lanthanide chelate complexes do not fluorescence with these ligands, a dissociation step must be carried out prior to detection.<sup>[74]</sup> Furthermore, once the immune reaction is complete, a so-called enhancement solution is added after washing, which leads to the dissociation of the chelate com-

plex and to the formation of fluorescent complexes [DELFLIA system (Dissociation Enhanced Lanthanide Fluorescence Immuno-Assay)]. The 1,3-diketones  $\beta$ -naphthoyltrifluoroacetone and pivaloyltrifluoroacetone are commonly employed.

The use of chelate complexes with trivalent lanthanide ions facilitated not only the development of highly sensitive immunoassays by time-resolved measurement, but also the simultaneous determination of several parameters, since Eu<sup>III</sup>, Tb<sup>III</sup>, Sm<sup>III</sup>, and Dy<sup>III</sup> complexes emit at considerably different wavelengths and have different fluorescence lifetimes. Several double determinations have been described.<sup>[73, 74]</sup> Eu<sup>III</sup>/Tb<sup>III</sup> chelates<sup>[76]</sup> or Eu<sup>III</sup>/Sm<sup>III</sup><sup>[76]</sup> complexes were used as label pairs. In simultaneous determinations of two parameters from each, the dissociation/enhancement principle was employed. A simultaneous and highly sensitive determination of more than two lanthanide labels is, however, not possible with the simple enhancement solutions.

Simultaneous multianalyte determinations are gaining interest,<sup>[74]</sup> since with the four lanthanide ions mentioned and specially developed enhancement solutions, so-called cofluorescence-based enhancement solutions (CFES),<sup>[79]</sup> four parameters could be determined simultaneously by using time-resolved fluorescence measurements.<sup>[73]</sup> The enhancement solutions formed in this way consist of a dissociation element, pivaloyltrifluoroacetone and Y<sup>III</sup>, as well as an element, 1,10-phenanthroline which enhances the fluorescence. Europium(III) and terbium(III) chelates with macrobicyclic ligands that contain  $\alpha,\alpha'$ -bipyridine or 1,10-phenanthroline units were already described earlier as efficient luminophores which act as molecular light transformers.<sup>[72d]</sup>

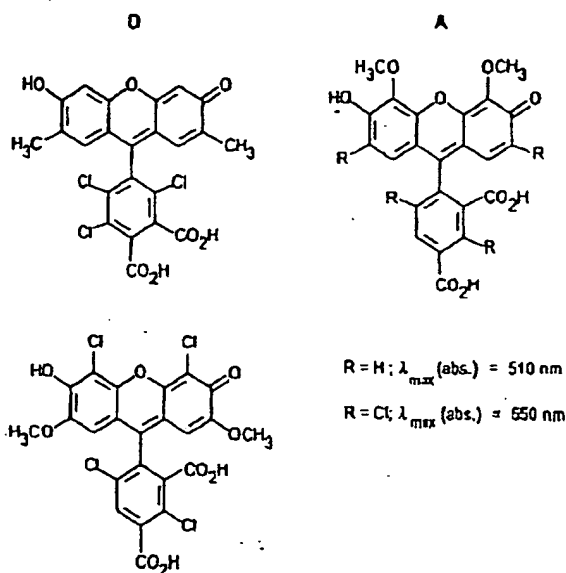
### 5.1.5. Fluorescence Energy Transfer

In the application of fluorescent labels for immunoassays, the principle of fluorescence-polarization, which facilitated development of homogeneous immunoassays, has already been mentioned. Another method, in which no separation of the unbound labeled molecules from the immunocomplexes is necessary, uses fluorescence energy transfers.<sup>[6b, 80]</sup> In this case an energy transfer from an electronically excited fluorophore (donor) to a neighboring acceptor dye molecule (quencher) occurs by dipole-dipole coupling. According to Förster<sup>[81]</sup> the efficiency of the energy transfer is indirectly proportional to the power 6 of the distance. With Förster's theory distance measurements in molecules, for example, can be obtained,<sup>[82]</sup> and for efficient energy transfer distances must not be greater than 10 nm. This condition is fulfilled in many antigen-antibody complexes. If, for example, the antigen is labeled with the donor and a specific antibody is labeled with the acceptor, quenching of fluorescence (of the donor) occurs in the immunocomplex. In a mixture of labeled and unlabeled antigens the fluorescence signal increases with the quantity of the unlabeled analyte to be determined.

The requirements for the fluorescent labels (donors) that should be employed in energy-transfer immunoassays are the same as those for fluorescent labels already mentioned. Furthermore, the choice of the donor-acceptor pair must be such that the emission spectrum of the donor and the absorption spectrum of the acceptor overlap well. In the beginning the use of the donor-acceptor pair fluorescein isothiocyanate/tetramethyl-

## REVIEWS

rhodamine isothiocyanate was described.<sup>[80a]</sup> Since interference from background signals such as background fluorescence from serum are particularly large in homogeneous immunoassay, standard labels such as fluorescein isothiocyanate, umbelliferones, or dansyl chloride certainly are of little significance. For the latter, in addition, the high sensitivity for background effects is disadvantageous. The same can be said for rhodamines such as tetramethylrhodamine. Due to higher absorption and emission wavenumbers, phycobiliproteins and lanthanide chelates are better suited as donors. With the latter, particularly in conjunction with time-resolved measurements, the development of more sensitive fluorescence energy-transfer immunoassays is possible. Also substituted fluoresceins with absorption and emission wavelengths greater than 500 nm (cf. Scheme 15) were used as donors.<sup>[80, 83]</sup>



Scheme 15. Fluorescein derivatives employed in fluorescent energy transfers: D = donor molecules, A = acceptor molecules.

The energy acceptors (quencher labels) should ideally fulfill the following conditions:<sup>[80a]</sup> a) high extinction coefficient of the emission wavelength of donor; b) no fluorescence during excitation in the absorption maxima of donor; c) good solubility in water in order to facilitate multiple labeling with the quencher (greater quenching effect); d) the smallest possible background interference in the absorption spectrum.

Since frequently used acceptors such as tetramethylrhodamines do not fulfill these requirements, new non-fluorescent fluorescein derivatives were described which form effective pairs with the donors in Scheme 15.<sup>[80, 84]</sup>

Fluorescent labeling and the principle of fluorescent energy transfers have recently also found application in the development of biosensors.<sup>[83]</sup> Detection can be based on the quenching of emission from the donor, new emission from the acceptor, or on the ratio of both emission wavelengths. One biosensor principle based on Langmuir-Blodgett films and fluorescence energy transfer with a coumarin derivative as donor and tetramethylrhodamine as acceptor was recently described.<sup>[80]</sup>

## 5.2. Chemiluminescent Labeling

A considerable difference between chemiluminescent detection systems and fluorescent labels is that former do not require the irradiation of the excitation light. With these chemiluminescent systems, in particular, in working with serum, the problems with high background signals, which are mainly responsible for the limited sensitivity of many methods with fluorescence detection, are prevented. However, in several chemiluminescent labels, complex systems comprising oxidation reagents, signal enhancer additives, and catalysts can likewise lead to an unacceptable high background signal, which, of course, has an adverse effect on sensitivity.<sup>[87]</sup> In the case of chemiluminescence detection in analysis systems, which above all are applied in medical diagnostics, compounds mainly from the following categories are employed:<sup>[9, 10-15, 17]</sup> luciferins in combination with the corresponding luciferases, cyclic arylhydrazides, acridinium derivatives, stable dioxetanes, and oxalic acid derivatives.

## 5.2.1. Bioluminescence

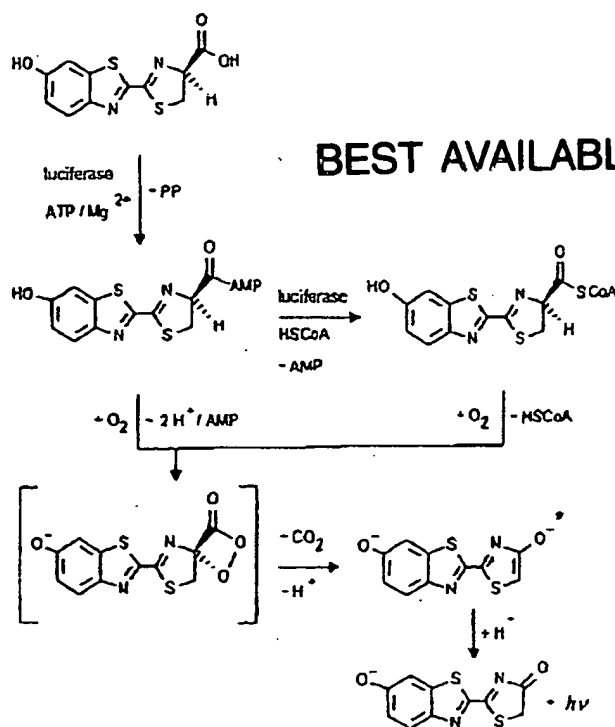
*The luciferin/luciferase system of the North American firefly*

One of the most well-known and most studied light systems in nature "operates" in the North American firefly (*Photinus Pyralis*). Although the mechanism of bioluminescence has been studied for more than 30 years, and the benzothiazole derivative luciferin became available synthetically and was structurally determined at the beginning of the 1960s, not all the details of the bioluminescence reaction have been elucidated. Since a more detailed description of this and other bioluminescent systems would go beyond the framework of this review article only the latest developments are described briefly.

As had been assumed for a long time<sup>[88a, b]</sup> and to a large extent proven at the end of the 1970s,<sup>[88c]</sup> the specific luciferase of the firefly catalyzes the oxidation of luciferin in the presence of ATP and magnesium ions (Scheme 16). Initially a complex is formed from the acyl-AMP species of luciferin and luciferase. In the presence of oxygen oxidation ensues to give excited oxyluciferin which returns to the ground state by emitting a photon.<sup>[15, 89, 90]</sup> In vivo the yellow-green emission ( $\lambda_{\max} = 565 \text{ nm}$ ) of the dianion was observed and in vitro an additional red emission ( $\lambda_{\max} = 615 \text{ nm}$ ) of the monoanion which was pH-dependent.<sup>[89, 90, 91]</sup> The oxidation proceeds presumably via a dioxetanone intermediate<sup>[11, 87, 92]</sup> which decarboxylates to furnish excited oxyluciferin.

To what extent one can view the often proposed dioxetanone as an intermediate or rather as a transition state is, as in the luminescent systems previously mentioned, still unclear. Instead of the dioxetanone intermediates in the oxidation of luciferins, acridiniumcarboxylic acid derivatives, and oxalic acid esters, the direct formation of excited products by charge-transfer can also be assumed during the decomposition of peroxide intermediates.<sup>[93]</sup> The mechanistic details cannot be emphasized within the framework of this review and interested readers should refer to the references [11, 87, 92, 93].

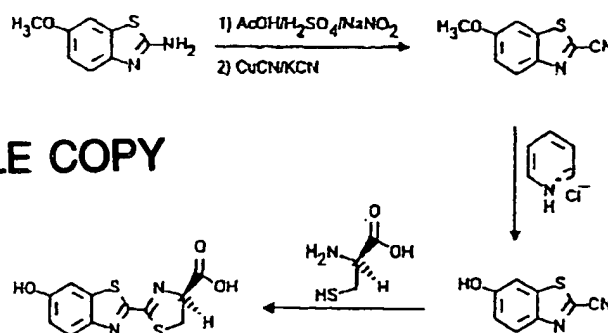
All in all, the light reaction of the firefly appears to be elucidated. However, the assumption, first made at the end of the 1950s,<sup>[94]</sup> that Coenzyme A also plays a role in the light reaction



Scheme 16. Chemistry of the light reaction of the firefly: PP = pyrophosphate; ATP = adenosine triphosphate, AMP = adenosine monophosphate, CoASH = coenzyme A.

has been confirmed in the past few years.<sup>[89, 95]</sup> Addition of the coenzyme may further improve the applicability of the firefly luciferin/luciferase system in the near future, since the intensity and duration of the light emission can be increased. The limiting factor for this method until now, in addition to the limited hydrolytic stability of luciferin and the sensitivity of luciferase, was, above all, the poor availability of the enzyme which could be extracted from fireflies. In the meantime the situation has fundamentally changed since the *Photinus Pyralis* luciferase, a protein with a molecular weight of 62 kD, can be expressed in bacteria, for example *E. coli*, by genetic engineering methods.<sup>[15, 89, 90, 97]</sup> The availability of genetically engineered luciferase and synthetic luciferin has now increased the expectation that this system, which with a quantum yield of up to 0.88 Einstein per mol is the most efficient of all known bio- and chemiluminescence systems, will be more widely applied than previously.

In the last thirty years essentially three synthetic pathways for the construction of firefly luciferin have been described, all of which proceed via the key intermediate 6-methoxybenzothiazole-2-carbonitrile (Scheme 17).<sup>[91, 98]</sup> The routes differ in the synthesis of the intermediate. Recently a new synthesis<sup>[99]</sup> has been published in which 6-methoxybenzothiazole-2-carbonitrile is obtained in one step by Sandmeyer cyanation of the commercially available 2-amino-6-methoxybenzothiazole (Scheme 17). The remainder of the synthetic pathway is already well-known: cleavage of the methyl ether and condensation with D-cysteine furnish the luciferin. In reference [99], earlier syntheses are also summarized. The oldest and until now most important application of the firefly luciferin/luciferase system is derived from the ATP-dependence of the bioluminescence reaction. Hence, a sen-



Scheme 17. Key step in the synthesis of the firefly luciferin.

sitive ATP determination can be carried out by using this system. ATP assays<sup>[100]</sup> are of interest above all in the screening for microorganisms in clinical microbiology in the areas of hygiene and nutrition.<sup>[101]</sup> A more recent application of steadily increasing significance is the use of the firefly luciferase gene as a reporter gene for the quantification of the gene expression in cells.<sup>[99, 102]</sup> Here a measurement of the light emission is made after addition of luciferin.

The use of luciferin derivatives,<sup>[103]</sup> which themselves are not substrates for luciferase, as substrates in enzyme immunoassays offers additional applications. After the luciferin has been released light emission is determined in the presence of luciferase.<sup>[103]</sup> In this way derivatives in which the phenolic hydroxyl group has been functionalized, such as D-luciferin-O-sulfate and -O-phosphate, can be cleaved by sulfatases or phosphatases. If the carboxyl group of the luciferin can be functionalized (methyl ester and phenylalanine and arginine amides have been described), the luciferin is released by carboxyesterases or carboxypeptidases.<sup>[103]</sup> Luciferin-O-phosphate as substrate for alkaline phosphatase, in comparison to the chromogenic substrate *p*-nitrophenylphosphate, facilitates a sixty-fold increase in sensitivity.<sup>[103a]</sup> In addition, D-luciferin-β-D-galactopyranoside as substrate for β-galactosidase was described.<sup>[104]</sup>

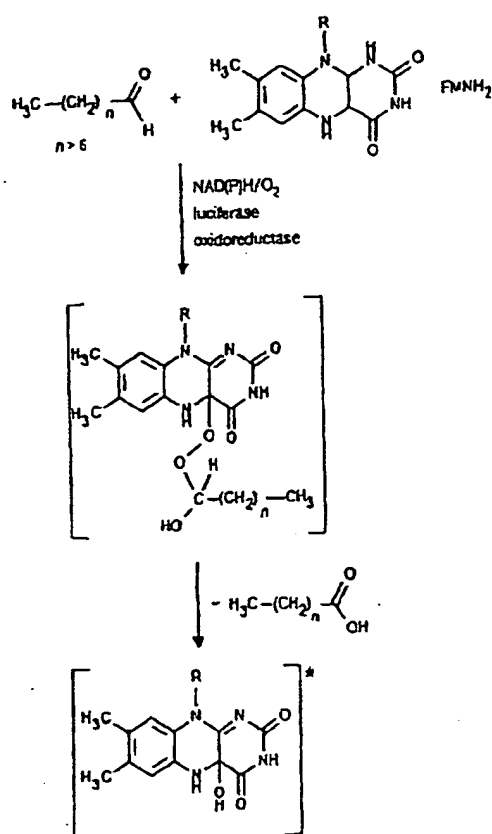
#### Bacterial luciferin derivatives/luciferases

In luminous bacteria such as *Photobacterium fischeri* and *Photobacterium phosphoreum*, light production ensues from the oxidation of long-chain aldehydes in the presence of reduced flavin mononucleotide (FMNH<sub>2</sub>), oxidoreductase, and bacterial luciferase.<sup>[15, 90, 97, 105]</sup> The intermediate is assumed to be a peroxide formed from a long-chain aldehyde and a flavin building block (Scheme 18). Depending on the bacterium, light emission occurs in the blue-green to yellow region of the spectrum with quantum yields of up to 0.3. The emitter is presumably a hydroxy derivative of FMN.<sup>[11, 15, 103]</sup> In vitro blue light (λ<sub>max</sub> = 492 nm) is emitted.<sup>[15, 103a]</sup> As with the firefly luciferase, several bacterial luciferases can be obtained by genetic engineering.<sup>[97]</sup>

The bioluminescence of bacterial luciferases can, in principle, be used to determine all the components that participate in the luminescence reaction, that is, NADH, NADPH, FMN, FMNH<sub>2</sub>, long-chain aldehydes, and oxygen.<sup>[103a]</sup> The possibility of determining the concentration of the extremely unstable FMNH<sub>2</sub> is theoretical. The use of bacterial luciferases is indeed still less widespread. The bioluminescent determination of long-



## REVIEWS



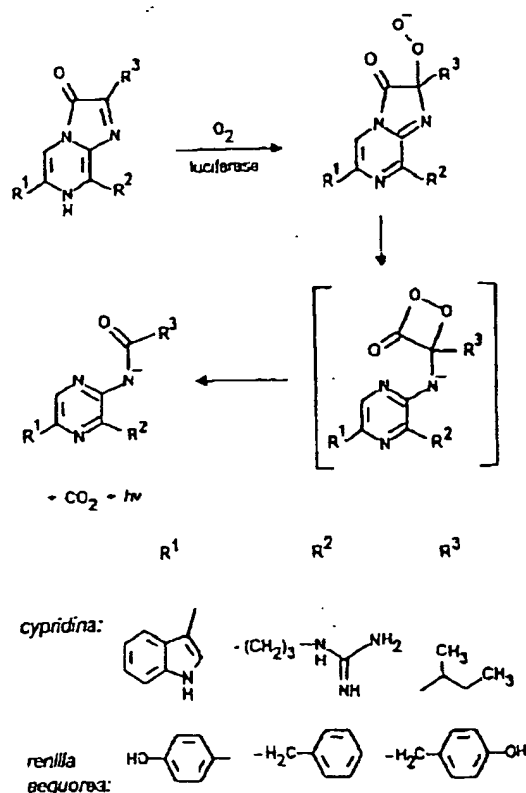
Scheme 18. Bacterial light systems.

chain aldehydes<sup>[105]</sup> and trace analysis of oxygen<sup>[105d]</sup> have some significance. A further field of application is in homogeneous DNA hybridization assays.<sup>[15, 107]</sup> The system for the bioluminescent determination of Papilloma viruses serves as an example.<sup>[107]</sup>

#### Luciferin derivatives with imidazopyrazine building blocks and photoproteins

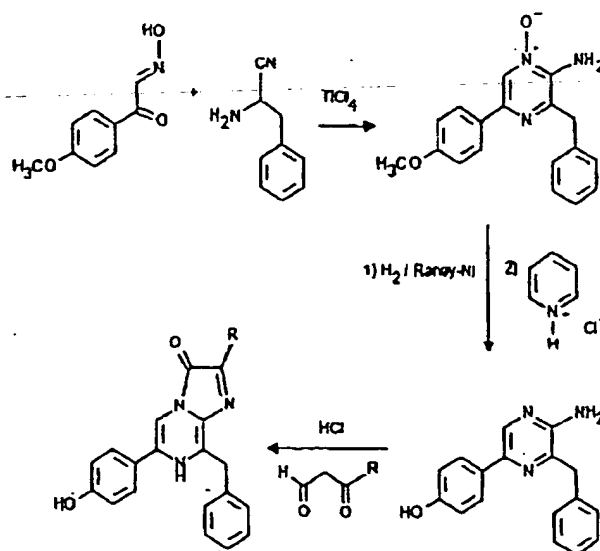
Bioluminescence occurs particularly frequently in marine life such as in crabs, jellyfish, mussels, sponges, fungi, and many fish.<sup>[9, 97b, 108]</sup> The luciferins of the mussel crab *Cypridina hilgendorffii*, sea pansy *Renilla reniformis*, and of the jellyfish *Aequorea aequorea* show a structural similarity (Scheme 19) which infers a common biosynthetic pathway.<sup>[92a]</sup> The luminescence mechanism is thought to consist of a catalytic oxygenation followed by ring closure to give an  $\alpha$ -peroxylactone. This intermediate decomposes with the formation of the emitter and carbon dioxide (Scheme 19). One should refer to the relevant discussions of the theories concerning the firefly luminescence mechanism in specialist literature.

The photoprotein Aequorin, which was obtained from the jelly fish *Aequorea victoria* in 1962, has aroused particular interest in recent years.<sup>[109]</sup> It consists of a complex of apoaquorin, coelenterazine (cf. Scheme 19), and molecular oxygen. Addition of calcium or strontium ions to the complex triggers light emission.<sup>[110a]</sup> One assumes that the binding of calcium ions to the protein induces the decomposition of the resulting oxygenated



Scheme 19. Structures of some imidazopyrazine luciferin derivatives and an outline of the peroxyfission mechanism.

chromophore. An additional luciferase is not necessary. The emitter was postulated to be the protein-bound anion of the chromophore (cf. Scheme 19).<sup>[110b]</sup> The active photoprotein is regenerated by incubation of the apoprotein with the coelenterazine in the presence of oxygen, ethylenediaminetetraacetate, and mercaptoethanol.<sup>[110a]</sup> In the meantime the apoprotein has become accessible by expression in *E. coli*.<sup>[111]</sup> The synthesis of the coelenterazine was described long ago,<sup>[112a, b]</sup> and in addi-



Scheme 20. Synthetic route for the construction of coelenterazines. R = CH<sub>3</sub>, C<sub>6</sub>H<sub>5</sub>, (CH<sub>2</sub>)<sub>2</sub>C<sub>6</sub>H<sub>5</sub> (n = 1–3), CH<sub>2</sub>C<sub>6</sub>H<sub>4</sub>OH.

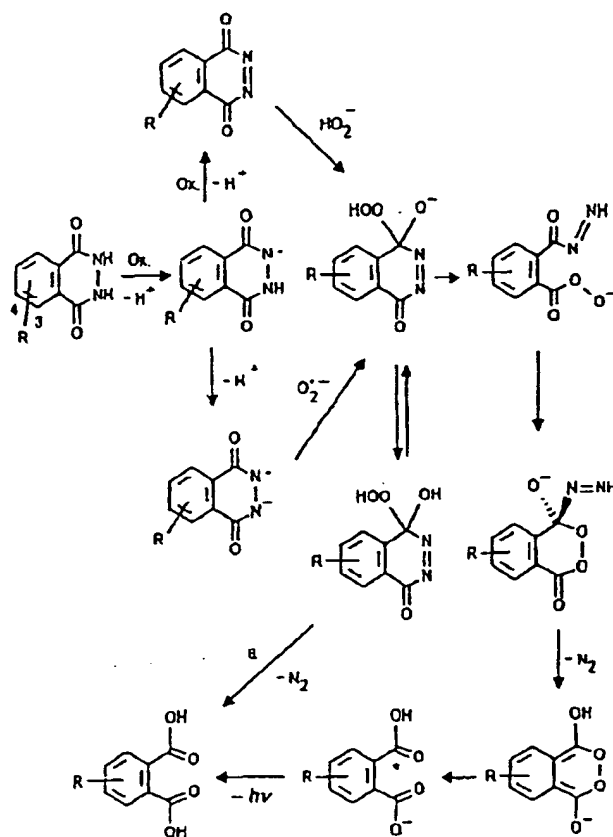
tion the synthesis of some systems with a modified structure was reported.<sup>[113c]</sup> The synthetic route is summarized in Scheme 20.

Aequorin can be used as a bioluminescent label after biotinylation. The triggering of light emission results from addition of calcium chloride solution. The label can also be detected in the attomol region. This label facilitated, for example, the development of a highly sensitive assay for salmonella determination. With regard to the sensitivity the test was shown to be clearly superior to other ELISA tests (see Section 3.1.2), even those with alkaline phosphatase as label and with chemiluminescent dioxetane AMPPD as substrate.<sup>[113a, b]</sup> In addition, the use of Aequorin in DNA and protein diagnostics<sup>[113d]</sup> and in the determination of serum glycoproteins has been described.<sup>[113e]</sup>

### 5.2.2. Cyclic Arylhydrazides

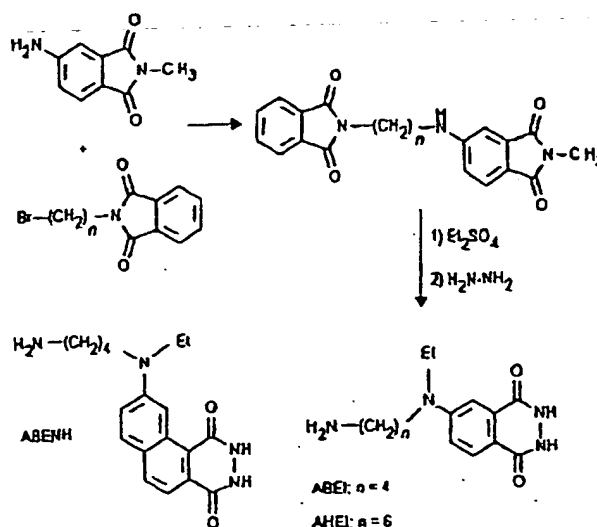
The chemiluminescence of luminol (3-aminophthalic hydrazide) was observed in 1928 in the form of blue light which was emitted during the oxidation with an alkaline solution of hexacyanoferrate(III) in the presence of hydrogen peroxide.<sup>[114]</sup> Luminol and carboxylic acid hydrazides have since been extensively studied. A wealth of reagents and catalysts can be employed in the oxidations of luminol and its derivatives. In organic, aprotic solvents, chemiluminescent reactions can be triggered by oxygen in the presence of a strong base. In aqueous solutions hydrogen peroxide is usually employed in the presence of catalysts such as peroxidases, hemin, and cobalt(II) salts.<sup>[104]</sup> Horseradish peroxidase (HRP)/H<sub>2</sub>O<sub>2</sub> is frequently employed. In the course of time, different reaction mechanisms have been discussed.<sup>[45, 51]</sup> A simplified reaction mechanism, which is only applicable for a one-electron oxidation and which affords the free luminol radical, is given in Scheme 21. According to a more recent review article it is suggested that the mechanism should be divided into two steps, formation of the key intermediate, an  $\alpha$ -hydroxyhydroperoxide, and its decomposition to the excited emitter. Whilst the formation of the hydroperoxide depends considerably on exact reaction conditions, decomposition of the key intermediate is only influenced by pH. Under these conditions the emitter appears to be the monoanion of aminophthalic acid. With other reagents, for example DMSO/base, the dianion of aminophthalic acid functions as the emitter. Intermediates in other proposed mechanisms are azaquinones, endoperoxides, and other peroxidic intermediates.<sup>[115b]</sup>

One of the oldest applications of luminol, which is still important today, is forensic blood analysis.<sup>[116]</sup> The application of luminol as a substrate for peroxidases in enzyme immunoassay has already been mentioned. Coupling reactions are necessary for the synthesis of labels for chemiluminescent direct labeling. The study of the chemiluminescent properties of luminol and isoluminol derivatives<sup>[117]</sup> had revealed that isoluminol only has approximately 10% of the quantum yield of luminol. Since, however, the chemiluminescent quantum yield of luminol decreases significantly<sup>[25, 37, 115, 117]</sup> if the primary 3-amino group is substituted, isoluminol derivatives, which are not sensitive with regard to substitution of the amino group, or the even more advantageous naphthalenedicarboxylic acid hydrazides which exhibit higher light yields,<sup>[21, 117a]</sup> were as a rule employed in the synthesis of arylhydrazide labels. The first label based on luminol, diazoluminol,<sup>[118]</sup> clearly showed the disadvantages out-



Scheme 21. Simplified mechanism of the chemiluminescence reaction of phthalic hydrazides. Ox = oxidising agent which affords the free luminol radical, for example, HRP. R = 3-NH<sub>2</sub>: luminol; R = 4-NH<sub>2</sub>: isoluminol. Since the regiochemistry of most processes is unclear, the position of the R residue remains open. a = dark.

lined and the quantum yield decreased to 1% of that of luminol. Considerable improvements were brought about by isoluminol derivatives in which the coupling group was introduced via the amino functionality.<sup>[117a]</sup> The structure and the synthesis of some important arylhydrazide labels are summarized in Scheme 22.

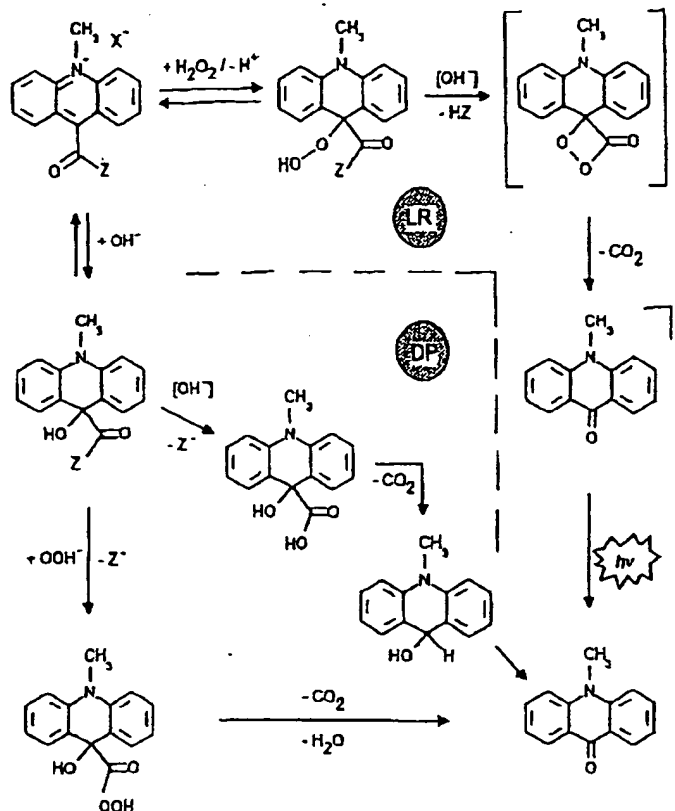


Scheme 22. Synthesis and structures of some arylhydrazide labels.

## REVIEWS

4-Amino-*N*-methylphthalic imide served as the starting material, which was successively alkylated with *N*-(bromoalkyl)phthalic imides and diethyl-sulfate. The subsequent hydrazinolysis of the bis-phthalic imide furnished the frequently used phthalic hydrazide labels ABEI and AHEI (*N*-amino-butyl- or *N*-aminohexyl-*N*-ethylisoluminol), which contain an amino functionality as the coupling group. The phthalic hydrazide label ABENH (*N*-aminobutyl-*N*-ethylnaphthylhydrazide) was obtained by a similar method. The starting material was dimethyl 7-amino-1,2-naphthalenedicarboxylate.<sup>(117b)</sup> Derivatives of these three labels which contain other reactive groups (isothiocyanate, *N*-hydroxysuccinimide ester) are also known. Conversion of AHEI to a derivative with a *N*-hydroxysuccinimide-reactive ester was shown previously in Scheme 3.

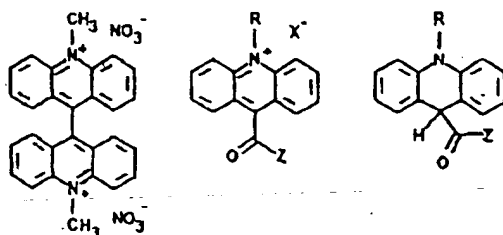
Arylhydrazide labels have and are finding broad application in immunoassays for chemiluminescent labeling of small and large molecules.<sup>(119)</sup> A distinct disadvantage is the considerable loss of the luminescent quantum yield of the label after the coupling. Furthermore, these labels are prone to interference since many components catalyze the luminescent reaction. In addition, the reagents which trigger light emission give rise to a large background signal which decreases the sensitivity.<sup>(125)</sup>



Scheme 24. Chemiluminescence mechanism and pseudo-base equilibrium of 9-acridiniumcarboxylic acid derivatives: DP = dark process, LR = light reaction.

## 5.2.3. Acridinium Compounds

The chemiluminescence of lucigenin [9,9'-bis(*N*-methylacridinium nitrate); Scheme 23] was reported in 1935.<sup>(120)</sup> But it was



Scheme 23. Examples of chemiluminescent acridinium compounds and acridanes: Z = leaving group.

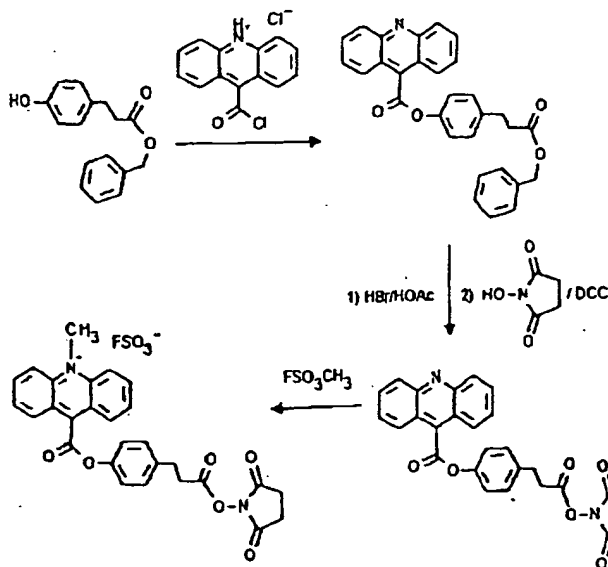
not until around thirty years later that studies led to other chemiluminescent acridinium compounds. These are specifically, 9-chlorocarbonylacridine hydrochloride,<sup>(121)</sup> 9-carboxy-10-methylacridinium chloride,<sup>(121)</sup> and 9-cyano-10-methylacridinium nitrate.<sup>(122)</sup> Today 9-acridiniumcarboxylic acid derivatives and acridanes are among the best-studied examples of chemiluminescent compounds. No additional reagents, apart from hydrogen peroxide and base, are necessary for the chemiluminescence of acridiniumcarboxylic acid derivatives. Quantum yields of up to approximately 0.05 can be attained with aryl esters;<sup>(11)</sup> higher still are the light yields which can be attained with

acridane aryl esters. The latter exhibit efficient chemiluminescence after treatment with a base in the presence of oxygen.<sup>(123)</sup> Only the acridiniumcarboxylic acid derivatives that have been more important for the development of luminescent labels will be considered in more detail below. The mechanism of their chemiluminescence shown in Scheme 24 can be considered to be elucidated as far as possible.

First of all, addition of hydrogen peroxide takes place at the electrophilic C-9 position of the acridinium unit. In the case of aryl esters—the leaving group Z then stands for phenolate—the corresponding hydroperoxides could be isolated and characterized.<sup>(123)</sup> After the addition of hydroxide spontaneous chemiluminescence resulted, usually as intense light flashes. A dioxetanone is often proposed as intermediate, which decomposes to give carbon dioxide and electronically excited *N*-methylacridone, the emitter. The transition to the ground state ensues by emission of a photon at a wavelength of approximately 430 nm. According to more recent studies,<sup>(123b)</sup> however, no dioxetanone seems to appear as a discrete intermediate. The final product of the light reaction, *N*-methylacridone, can also be formed by other pathways in dark reactions. An important prerequisite for degradation in the dark is the well-known<sup>(124)</sup> pH-dependant pseudo-base equilibrium of acridinium compounds.<sup>(125)</sup> The reactions, which for the phenyl ester were studied more accurately in a flow system,<sup>(124)</sup> are integrated into Scheme 24. It is immediately clear that both the light reaction and the dark process are dependent, for instance, on the properties of the leaving group Z. Other important factors include the peroxide concentration

and the pH value.<sup>[126, 127]</sup> McCapra et al. showed that for effective chemiluminescence the pK value of the conjugate acid of the leaving group should be less than 12—the pK value of hydrogen peroxide.<sup>[108]</sup> Since a good label should exhibit a high chemiluminescent yield in addition to a high stability in the labeled reagent, the discovery of suitable compounds can be equated to a fine balancing act, ending in a compromise between light yield and stability.

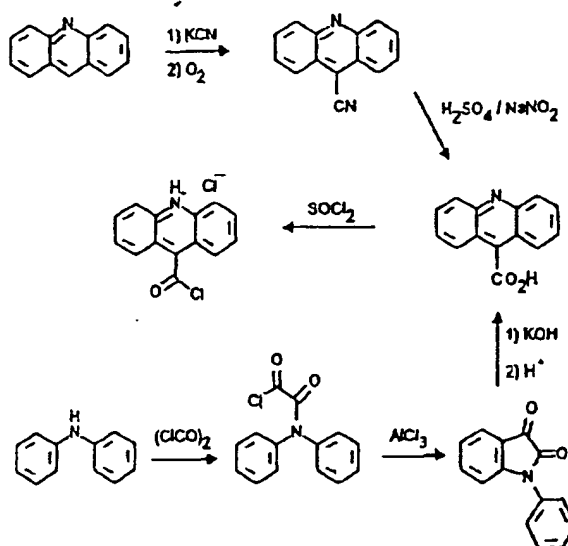
The first attempts to use 9-acridiniumcarboxylate as a label in immunoassays were reported at the beginning of the 1980s. In this an attempt was made to couple aryl esters, which contained free carboxyl groups, to proteins, after activation of the carboxyl groups. These labeling experiments had only limited success.<sup>[128]</sup> It was not until a phenyl *N*-methylacridinium-9-carboxylate, containing a hydroxysuccinimide ester on the phenyl group as the reactive group for coupling to proteins, was used that successful labelings could be carried out.<sup>[126, 129]</sup> The synthesis of this prototype of a chemiluminescent label based on a 9-acridiniumcarboxylic acid derivative,<sup>[25, 126, 128–130]</sup> the so-called Woodhead label, is summarized in Scheme 25.



Scheme 25. Synthesis of an acridinium ester label.

An important precursor for this and other labels discussed later was 9-acridinecarboxylic acid, whose synthesis is shown in Scheme 26. One reaction route starts from acridine and proceeds via 9-cyanoacridine to give the carboxylic acid.<sup>[131]</sup> In another synthetic method 9-acridinecarboxylic acid is formed from diphenylamine, which is acylated with oxalyl chloride and is cyclized by using aluminum trichloride to give the *N*-phenylisatine.<sup>[132]</sup> The synthesis of substituted acridinecarboxylic acids from substituted *N*-arylisatines has also been described;<sup>[133]</sup> however, it appears to work only on a very small scale.

Since the acridinium ester label mentioned was not sufficiently stable for development of commercial chemiluminescence immunoassays<sup>[129, 133]</sup> (hydrolysis of the ester bond (cf. degradation in the dark in Scheme 24) results in a too rapid a decrease of activity in conjugates), different research groups have been



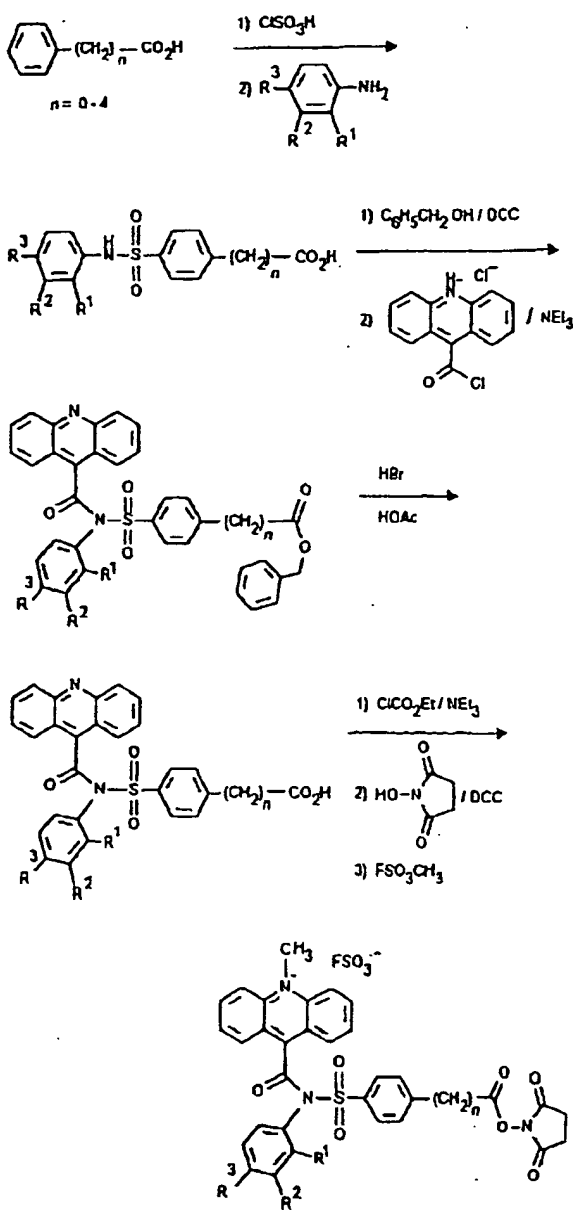
Scheme 26. Possible synthetic routes to 9-acridinecarboxylic acid.

looking for more stable labels based on acridiniumcarboxylic acid. One solution to the problem involved the steric shielding of the ester bond and the C-9 position of the acridine unit, for example, by methyl groups in the 2,6-position of the aryl ring.<sup>[134]</sup> In addition, other 9-acridiniumcarboxylic acid-(2,6-substituted)aryl esters were described.<sup>[136]</sup> Here the reactive group can also be bound through a spacer to the acridinium system. To complete the picture one should not forget to mention that aryl *N*-methylphenanthridinium-6-carboxylates have been described as chemiluminescent labels.<sup>[135, 137]</sup>

Other research groups have attempted to improve the properties of the acridiniumcarboxylic acid derivatives in comparison to those of the aryl esters, by varying the leaving groups. Thiol esters, indeed, brought about progress in as far as the light yield is concerned, but not, however, as regards to hydrolytic stability.<sup>[138]</sup> A significant improvement of the stability and very good chemiluminescence quantum yields were achieved when *N*-sulfonylamide anion was used as the leaving group instead of phenoxide.<sup>[138–141]</sup> In this class of compounds the spectrum of properties can be influenced much more specifically than for the acridinium ester labels by tailored variations in the structures. For instance, the solubility in water (an important parameter) can be significantly improved by the introduction of suitable substituents.<sup>[139, 142]</sup> The synthesis of *N*-methylacridinium-9-(*N*-sulfonyl)carboxamide labels is summarized in Scheme 27.

In addition, the hydrolysis behavior and the kinetics of emission can be varied to a certain extent.<sup>[142, 143]</sup> Although phenols and sulfonamides have similar pK values, labels with the latter are usually considerably more stable. This may be attributed to a combination of steric shielding effects and electronic stabilization,<sup>[127, 143]</sup> through which the dark reaction pathways described are minimized. It is assumed that in *N*-sulfonylcarboxamides there is an increased bond order of the C–N bond in comparison to the ester C–O bond. This is also evident from the frequencies of the carbonyl stretching vibration in the IR spectrum.<sup>[143]</sup>

Labels based on 9-acridiniumcarboxylic esters and acridinium-9-(*N*-sulfonyl)carboxamides have, in the meantime, found

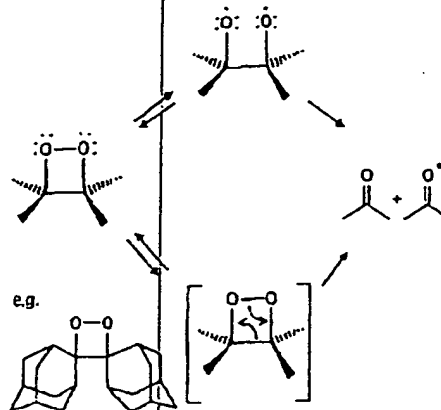

 Scheme 27. Synthesis of acridinium-9-(*N*-sulfonyl)carboxamide labels.

broad application in commercial immunoassays (see Section 6).<sup>(119c, 144)</sup> Up until now only a few applications for other acridinium derivatives have been described. For example, lucigenin can be used in micellar chemiluminescence assays for the determination of reductants (ascorbic acid, uric acid, glucose, and fructose).<sup>(145)</sup> The micelles are necessary to improve the solubility of lucigenin.

#### 5.2.4. Dioxetanes

Dioxetanes have for a long time been regarded as merely having curiosity value in the laboratory. Use of the extremely unstable compounds in reagents for diagnostics was not considered. It was not until after adamantylideneadamantane-1,2-dioxetane (an extremely stable compound due to the steric shielding prepared by W. Adam et al.<sup>(146)</sup> in 1972) became known that the

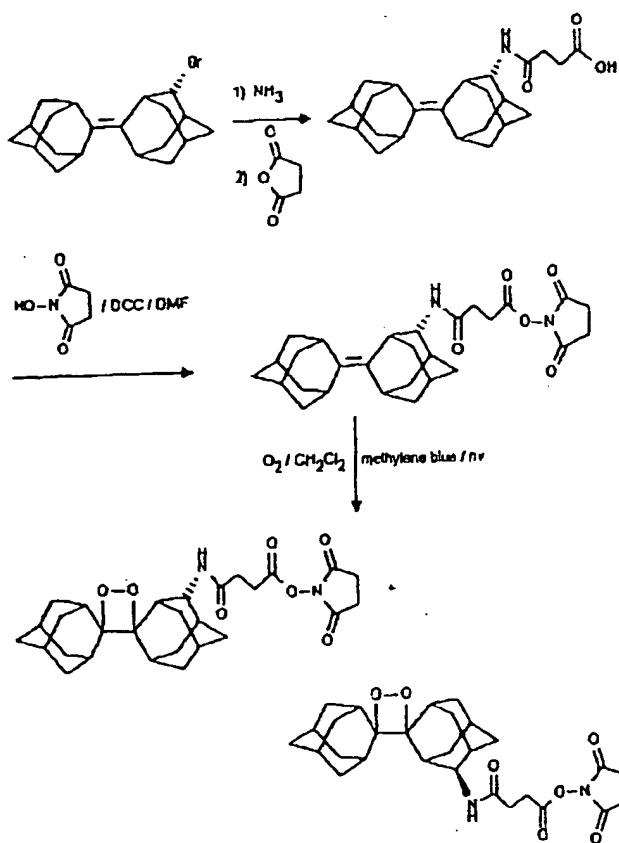
development of dioxetane labels really took off. Light emission could only be triggered thermally for stable dioxetanes of the type mentioned which exhibit a half-life of greater than 20 years at room temperature. In the thermolysis two molecules of adamantone are formed, partly in the  $S_1$  state and partly in the  $T_1$  state (Scheme 28). In principle, cleavage can occur according



Scheme 28. Simplified representation of the decomposition reaction of dioxetanes.

to a diradical or a concerted mechanism.<sup>(87)</sup> A stepwise pathway, involving homolysis of the O-O bond and formation of a diradical, has been proposed for the decomposition of the stable dioxetanes.<sup>(147)</sup> The light emission results from the deactivation of the  $S_1$  excited species. Dioxetanes, which, in addition to the steric stabilization by only one adamantyl group, still contain a substituent of low oxidation potential, mostly aryloxy, undergo a different decomposition mechanism. This decomposition route is triggered by cleavage of the O-O bond and by an electron transfer from the oxidizable group into the antibonding orbital of the peroxide bond (CIEEL mechanism, chemically initiated electron exchange luminescence).<sup>(91c, 148-155)</sup> This mechanism for the dioxetanes mentioned which are substrates for enzyme labels is discussed in Section 4.

Functionalized adamantylideneadamantanes that contain a reactive group bound to a spacer have been described as a label for thermochemiluminescent immunoassays<sup>(156)</sup> (Hummelen et al., 1986). The synthesis of one such label is given in Scheme 29. The starting material is adamantylideneadamantane, which can be obtained in two steps from adamantanone.<sup>(157)</sup> In the last step of the synthesis, sensitized (methylene blue) photooxygenation, a mixture (ca. 1:1) of two dioxetane isomers results, which was used in this form in the labeling experiments. The overall yield of the seven-step synthesis, starting from adamantanone is 50%. The triggering of luminescence results from heating the sample adsorbed onto aluminum oxide for a short time at 240°C. An apparatus for measuring thermochemiluminescence has also been described.<sup>(156)</sup> Since the efficiency of the direct chemiluminescence of adamantylideneadamantane-1,2-dioxetane is  $1 \times 10^{-4}$  ( $6 \times 10^{19}$  photons per mol)<sup>(158)</sup> under optimal conditions (only 1% of that of luminol), an increase in the energy transfer to a good fluorescent dye is necessary. Bovine serum albumin conjugates with the dioxetane label and 9,10-diphenylanthracene, which, in turn, have been used as labels in

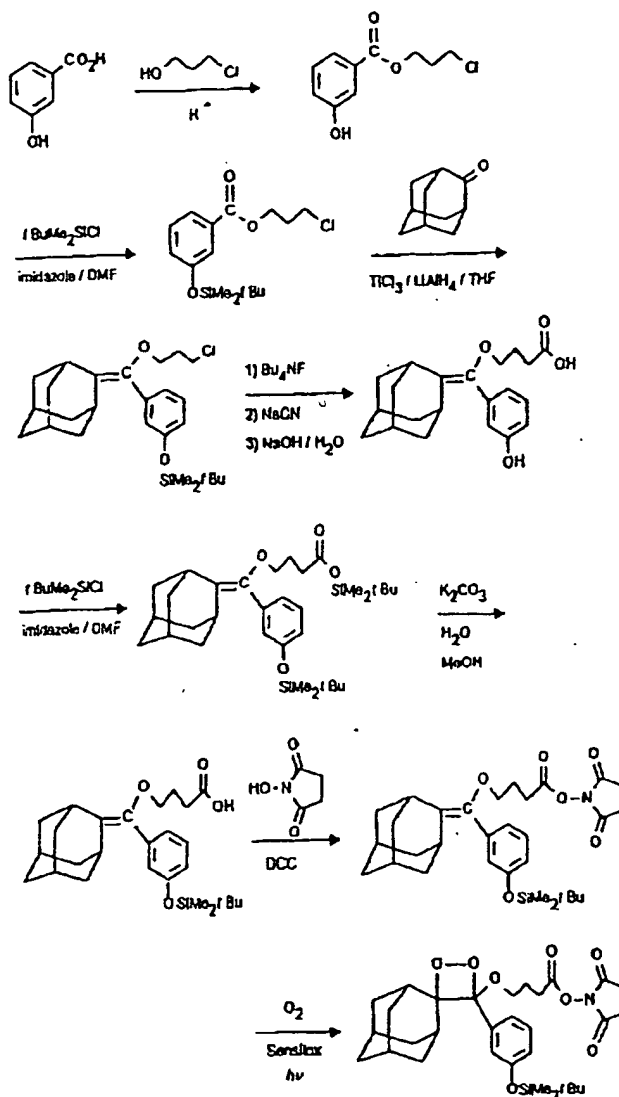


Scheme 29. Synthesis of a thermochemiluminescent dioxetane label. DMF = dimethylformamide.

Fluorescence amplified thermochemiluminescence immunoassay (FATIMA), have been described. The first assays, for example, for the tumor marker CEA have been described, but the thermochemiluminescence principle appears to be altogether too costly for wider commercial use. The search was then started for model compounds for thermally somewhat less stable dioxetane labels, which could be activated at approximately 150 °C.<sup>[159]</sup> For example, 9-xanthenyldioxetane decomposes at around 100 °C (Hummelen et al., 1988). The light emitted from this compound corresponds to the emission from adamantane. Other monoadamantyldioxetane derivatives of the xanthene, naphthalene, and phenyl series, which can be triggered enzymatically and chemically and in which the olefinic starting material contains a methoxy substituent to facilitate dioxetane synthesis, have been described (Schaap et al., 1987).<sup>[147c, 47d]</sup> Shortly afterwards AMPPD, already mentioned in Section 4, was reported. In contrast to the above-mentioned thermal decomposition, the emitter in the CIEEL decomposition of adamantyldioxetane, which can be triggered enzymatically or chemically (cf. Scheme 10), is chiefly an excited aryloxy anion.

Thus, in less than twenty years since their discovery, dioxetanes have developed from merely being laboratory curiosities to being stable derivatives employed worldwide in immunological and biochemical analysis. The development has not yet reached an end. Until a short time ago the 1,2-dioxetanes, which can be triggered enzymatically or chemically, were not known as classical labels with a reactive group for coupling to molecules to be

labeled, however, recently also such dioxetane labels for direct labeling methods were introduced.<sup>[14, 47a, 160]</sup> The synthesis of a selected label is outlined in Scheme 30. Apart from the silyloxy

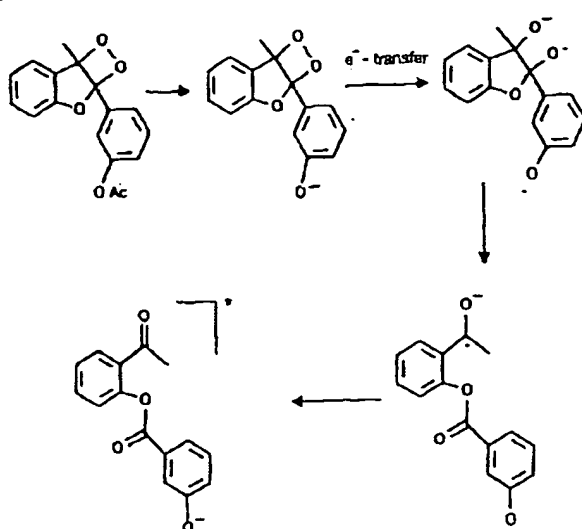


Scheme 30. Dioxetane labels for chemiluminescent direct labeling which can be triggered chemically. Sensitox = polymer-supported Rose Bengal.

group shown which can be cleaved to trigger luminescence, other substituents can also be employed, for example, phosphate and galactosyl groups. In addition to the hydroxysuccinimide esters shown other common reactive groups should also be considered. A label with a biotin anchor and its use for labeling proteins and antibodies has also been described.<sup>[147d]</sup> Further applications of this relatively new label are as yet unknown, but judging from the high quantum yields (0.20–0.25 in DMSO)<sup>[14]</sup> they will presumably not be long in coming.

Finally in this section on dioxetanes another new class of relatively stable dioxetanes should be mentioned namely, phenyl-substituted benzofuran-1,2-dioxetanes.<sup>[14, 92a, b]</sup> These give quantum yields (up to  $4 \times 10^{-6}$ ) similar to those of the enzyme substrates described earlier. The acetoxy-substituted

## REVIEWS



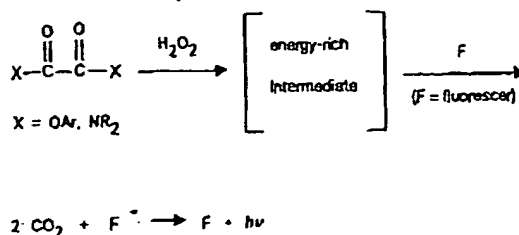
Scheme 31. Structure and decomposition of a benzofurandioxetane.

compound shown in Scheme 31 decomposes, presumably base-induced, according to the CIEEL mechanism. For analogous siloxy-substituted compounds decomposition can, as with similar adamantyl systems, be triggered by fluoride ions.<sup>[92]</sup>

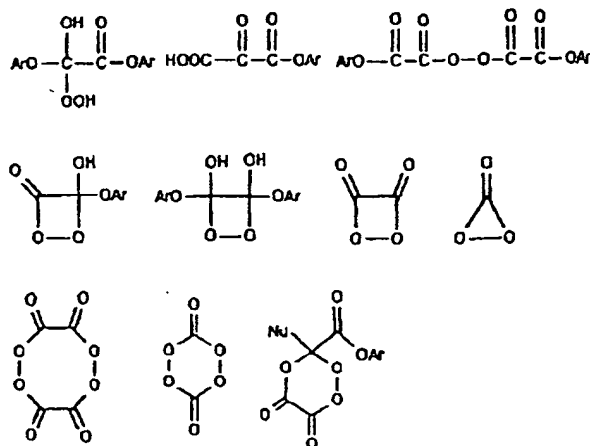
## 5.2.5. Oxalic Acid Derivatives

Oxalic acid derivatives such as oxalyl chloride,<sup>[161, 162]</sup> certain oxalic acid anhydrides,<sup>[163]</sup> diaryl esters,<sup>[164]</sup> and oxamides<sup>[165]</sup> are among the synthetic molecules which exhibit the highest chemiluminescence quantum yields (up to 0.5).<sup>[14]</sup> Intense luminescence is observed in the presence of a fluorescent dye during the oxidation of oxalic acid derivatives with hydrogen peroxide. Despite extensive studies the complete mechanism of peroxyoxalate chemiluminescence is still not fully understood. A dioxetanedione is often proposed as the energy-rich intermediate which forms the oxalic acid derivative by reaction with hydrogen peroxide (Scheme 32). However, according to the results of more recent work, this highly strained intermediate is not formed;<sup>[93a]</sup> a large number of possible intermediates is shown in Scheme 32.<sup>[14, 166-169]</sup> As has already been considered in other mechanisms, reaction pathways involving electron transfer or energy transfer are discussed.<sup>[14]</sup> Finally, either excited carbon dioxide is formed which activates the fluorescent dye by energy transfer, or a charge-transfer complex is formed which decomposes to give CO<sub>2</sub> and a fluorescent dye molecule in the excited state. The luminescence of these systems is relatively long lasting and the emission color can be controlled by choice of dye. The best known application of this are the Cyalume light sticks.<sup>[170]</sup>

Oxalic acid esters or oxamides cannot be considered for the development of luminescent labels for diagnostic purposes because the solubility of the oxalates and fluorescent dyes requires the use of organic solvents: the compounds are hydrolyzed quickly in aqueous solutions.<sup>[14]</sup> However, oxalates are employed in chemiluminescence detectors in HPLC or flow injection analysis.<sup>[14, 171]</sup> The appropriate systems allow, inter alia, analysis of environmental toxins, drugs, amino acids, fatty acids, and amines with detection sensitivities ranging from the nanogram



Selection of postulated energy-rich intermediates:



Scheme 32. Mechanism of chemiluminescence for oxalic acid derivatives.

into the attomol region. Another interesting area of application of oxalate chemiluminescence in diagnostics is the quantitative determination of oxalic acid in urine with detection limits as low as 10 nmol L<sup>-1</sup>. For this purpose the oxalic acid present is treated with carbodiimide in the presence of hydrogen peroxide and a fluorescent dye. The light emission measured is proportional to the concentration of oxalic acid.<sup>[172, 173]</sup> Even the determination of porphyrins in urine is possible with this method. In this case a fluorescent dye is not necessary because the porphyrins themselves act as sensitizers.<sup>[174]</sup>

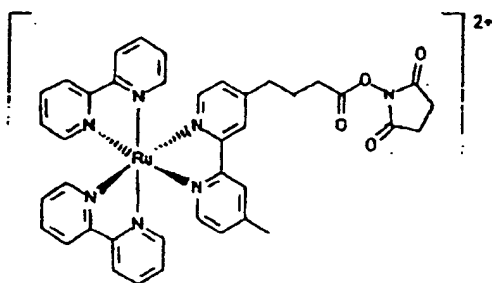
## 5.2.6. Electrochemiluminescence

The triggering of chemiluminescence by electrochemical processes has been known for a long time but has only gained practical significance in recent years.<sup>[175]</sup> Radicals produced electrochemically play a significant role in these processes and the electrochemical excitation of luminol/hydrogen peroxide mixtures has been studied in detail.<sup>[176]</sup> Luminescence is observed with potentials greater than 0.5 V. A mechanism similar to the one presented in Scheme 21 is assumed for potentials of up to 0.7 V. Diazaquinone produced electrochemically reacts with hydrogen peroxide. At higher potentials (1.2 V) the reaction is considerably more complex since the amino group of luminol is involved in oxidation processes.<sup>[176]</sup> An electrochemical detector based on this system can be used for the determination of hydrogen peroxide.

In addition, the electrochemical processes of acridinium compounds have been investigated.<sup>[177]</sup> Whilst lucigenin is reduced at -0.3 V and gives rise to luminescence in a subsequent reaction of the radical, acridinium esters show no activity in the range +1

to IV. However, the luminescent reaction of the acridinium esters can be initiated electrochemically by hydrogen peroxide produced from oxygen. This principle facilitated development of detectors for labeled analytes without the need for additional reagents. Lysin labeled with the first acridinium ester described earlier could be detected with a sensitivity of 10 fmol.<sup>1177</sup>

The chemiluminescence of ruthenium(II) chelate complexes has been known for a long time.<sup>1178, 1179</sup> likewise the electrochemically generated luminescence from trisbipyridine-ruthenium(II) chelates.<sup>1180</sup> However, the labeling of haptens, proteins, and nucleic acids with ruthenium(II) chelates was only described recently.<sup>1181</sup> The ruthenium complex, which is shown in Scheme 33, uses a hydroxysuccinimide ester sub-



Scheme 33. Structure of an electrochemiluminescent label based on a trisbipyridine-ruthenium chelate

stituent as the reactive group. The advantages of the label cited are high stability, relatively low molecular weight, high solubility in water, and high sensitivity—the detection limit of the label is 200 fmol L<sup>-1</sup>. Multiple labeling of proteins and oligonucleotides are possible without being detrimental to immune reactivity, solubility, or ability of the conjugate to hybridize. In the electrochemical reaction, [Ru(bpy)<sub>3</sub>]<sup>2+</sup> (bpy = bipyridine) is first oxidized to [Ru(bpy)<sub>3</sub>]<sup>•+</sup> on the electrode surface. Simultaneously, the tripropylamine (TPA) present in a large excess is likewise oxidized to a radical cation TPA<sup>•+</sup> which spontaneously cleaves a proton. In the reaction of the strong oxidizing reagent [Ru(bpy)<sub>3</sub>]<sup>•+</sup> with the radical TPA<sup>•</sup>, a strong reducing agent, a [Ru(bpy)<sub>3</sub>]<sup>2+</sup> complex is formed in the electronically excited state which returns to the ground state by emission of a photon at 620 nm. The ruthenium(II) complex can re-enter the cyclic process, which automatically causes amplification of the signal.

In addition to an electrochemiluminescent analyser,<sup>1179b</sup> immunoassays,<sup>1179a</sup> and genetic probe tests<sup>1179c, 1180</sup> with the, in the meantime, commercially available ruthenium label have been described. The available data is insufficient to evaluate the suitability of electrochemiluminescent detection in diagnostic practice.

## 6. Applications

### 6.1. Immunoassays

#### 6.1.1. Introduction

When Yalow and Berson developed the first radioimmunoassay for the in vitro determination of insulin in 1959,<sup>111</sup>

they surely could not have envisaged that their method in this or in some other form would become the most important analytical tool of medicinal in vitro diagnostics. It is difficult to overlook the wealth of substances, whose concentrations are today routinely determined in clinical laboratories with immunoassays. A number of these analytes are listed in Table 2 according to diagnostic indications.

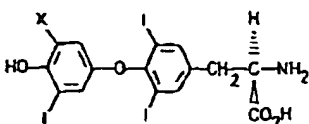
Table 2. Some analytes which are routinely determined by immunoassay in different diagnostic areas.

<i>Diseases of the thyroid gland</i>	<i>Gastrointestinal tract</i>
TSH (thyroid stimulating hormone, thyrotropin)	calcitonin
T <sub>3</sub> (triiodothyronine)	C-peptide
FT <sub>3</sub> (free T <sub>3</sub> , i.e. not bound to binding protein)	gastrin
T <sub>4</sub> (tetraiodothyronine)	insulin
FT <sub>4</sub> (free T <sub>4</sub> , i.e. not bound to binding protein)	anti-insulin antibody
YBG (thyroxine binding globulin)	pepsinogen
Tg (thyroglobulin)	trypsin
Anti-Tg (autoantibody against Tg)	tryptic neostatal
TPO (thyroidal peroxidase)	vitamin D
Anti-TPO (autoantibody against TPO)	
TRAK (autoantibody against the TSH receptor)	
<i>Tumour growth controls</i>	<i>Hypertonia and nephrology</i>
AFP (α-fetoprotein)	aldosterone
CA 50 (CA = cancer antigen)	androstenedione
CA 125	angiotensin I (renin)
CA 15-3	cortisol
CA 19-9	DHEA (dehydroepiandrosterone)
CA 22-4	17 α-OH-progesterone
CA 75-1	
calcitonin	<i>Cardiology</i>
CEA (carcinoembryonic antigen)	digoxin
cya 21-1	myoglobin
ferritin	
HCG (human choriongonadotropin)	<i>Connective tissue diseases</i>
β <sub>2</sub> -microglobulin	luminin
NCAM (neural cell adhesion molecule)	NC1 (N-terminal collagen 1)
NSE (neuron specific enolase)	PIIIP (procollagen-III-peptide)
osteocalcin	7S-collagen
PAP (prostatic acid phosphatase)	
prolactin	<i>Infectious diseases</i>
PSA (prostatic specific antigen)	HBsAg (hepatitis B surface antigen)
SCC (squamous cell carcinoma antigen)	HSV antigen
TATI (tumour-associated trypsin inhibitor)	p24 antigen (HIV antigen)
Tg (thyroglobulin)	rotavirus antigen
TPA (tissue polypeptide antigen)	antibodies against:
	- FSME virus
	- HBsAg
	- HIV1
	- HIV2
	- HSV
<i>Pregnancy</i>	- measles virus
AFP (α-fetoprotein)	- rotavirus
estriol	- rubella virus
HCG (human choriongonadotropin)	- toxoplasma gondii
progesterone	- varicella Zoster
<i>Sexual functions</i>	<i>Inflammatory processes</i>
FSH (follicle stimulating hormone, follitropin)	α-antitrypsin
LH (luteinizing hormone, lutropin)	CRP (C reactive protein)
progesterone	α <sub>2</sub> -macroglobulin
17α-OH-progesterone	α <sub>2</sub> -acid glycoprotein (Orosomucoid)
prolactin	
testosterone	

The success of immunological assays is owed primarily to their high specificity and sensitivity; antibodies, which are employed in immunoassays as detection reagents, can "recognize" at the molecular level smallest structural differences (much cited "lock-and-key" principle). For example, an antibody raised



against the thyroid hormone thyroxine binds with high affinity (equilibrium constants usually are of the order of  $10^{10}$ –



Scheme 34. Structure of the thyroid hormones T3 (X = H, 3,3',5-triiodo-L-thyronine) and T4 (X = I, 3,3',5,5'-tetraiodo-L-thyronine, L-thyroxine).

$10^{12}$  L mol<sup>-1</sup>), whereas triiodothyronine which differs by only one iodine atom is not recognized (Scheme 34).

As a result of this impressive specificity practically all substances with a molecular weight of greater than 100 D even in compli-

cated liquids such as serum can be determined exactly without prior separation of similar substances. Modern labels can even be traced into the attomolar range (1 amol =  $10^{-18}$  mol). Thus, by labeling antibodies with such labels, the substances to be analyzed can be quantified exactly to the femtomolar range (1 fmol =  $10^{-15}$  mol). A current listing of almost 500 literature references can be found in "Bioluminescence and Chemiluminescence Literature - Immunoassays and Blotting Assays" by O. Nozaki et al.<sup>(107)</sup>

### 6.1.2 Categories

Immunoassays can be divided into different groups.

**Group I:** Competitive immunoassays with analyte tracer:

This group concerns assays in which the detection reagents are an antibody specifically directed against the substance to be determined and an analyte derivative which carries the label (analyte derivative tracer, usually abbreviated to analyte tracer). The analyte tracer should not be too structurally different

from the analyte (which under the circumstances only exists in the presence of the label) that it no longer is recognized by the antibody. Furthermore, immunoassays of this group are competitive assays, that is the analyte and analyte tracer compete for a small number of antibody binding sites in an equilibrium reaction (Fig. 3a). The lower the concentration of the analyte-sample to be analyzed, the more antibody-analyte tracer complexes can form as a result. The analyte concentration of an unknown sample can be determined exactly by using a calibration curve drawn up from samples of known concentration (Fig. 3b). As is apparent from Figure 3a, selective measurement of the signal emitted from the antibody-analyte tracer complex requires prior separation of uncomplexed analyte tracer. Separations of this kind are dealt with in Section 6.1.3.

**Group II:** Competitive immunoassays with antibody tracer:

As in Group I this is also a competitive process. The only difference is that of the two detection reagents (antibody, analyte derivative) it is not the analyte derivative that carries the label but the antibody (antibody tracer also called tracer antibody). As can be seen from Figure 3c, the concentration of the analyte to be determined correlates with the concentration of the antibody tracer-analyte derivative complex after adjusting the equilibrium (Fig. 3d). For the separation of the two labeled complexes which is also necessary in this case see Section 6.1.3.

**Group III:** Sandwich assays:

Instead of the analyte derivative in competitive assays, in this case a second antibody is the detection reagent. The label is situated on one of the two antibodies. Both antibodies bind to the analyte at different sites (epitopes) and thus form a sandwich complex. An excess of the two antibodies is employed in order to shift the equilibrium in favor of the sandwich complex (Fig. 3e). The favorable equilibrium position in this type of assay due to the excess of reagent leads to a considerably higher sensitivity of detection. Whilst, for example, a competitive assay for the determination of the thyroid hormone thyrotropin is capable of detecting an analyte concentration of about 1–2 fmol mL<sup>-1</sup>, the lower detection limit in the case of a comparable sandwich assay is about 0.1–0.2 fmol mL<sup>-1</sup>. The sandwich strategy achieved its breakthrough when it became possible to obtain pure uniform antibodies in virtually any quantity. (Monoclonal antibodies, Nobel Prize, 1984 for Köhler and Milstein).<sup>(102, 103)</sup>

About the only disadvantage is the limited applicability of this type of assay: small analytes ( $M < \text{approx. } 5 \text{ kD}$ ) are excluded, since two antibodies cannot bind simultaneously for steric reasons. In the case where the analyte is an antibody (cf. listing in Section 6.1.1; indication: infectious diseases), the sandwich assay can be applied in a slightly modified form: an antigen plays the part of one of the two detection antibodies, thus, for example, a virus particle, against which the analyte antibody is directed. In this case, the resulting sandwich complex consists of antigen, analyte antibody, and detection antibody; the antigen corresponding to its complementary structure binds to the recognition site of the analyte antibody and is, thus, responsible for the specificity of the detection. For the detection antibody it suffices if this is directed against the structure element of the analyte antibody, although this is standard for all

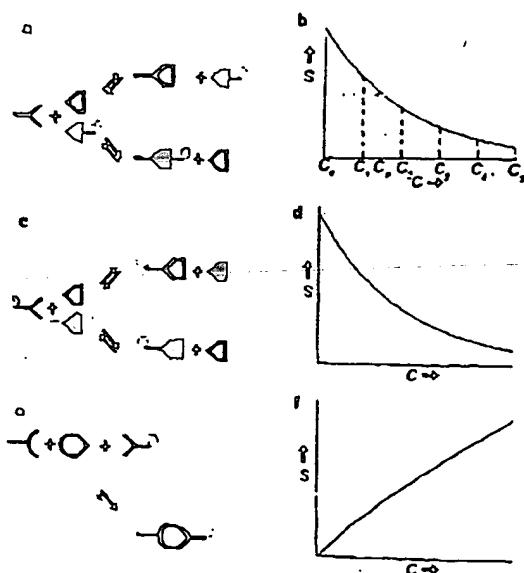


Fig. 3. Principle of the three most important immunoassay methods (left) with the corresponding calibration curves (right). Left: blue = antibody, green = analyte (open) and analyte derivative (closed), red = label. Right: c = concentration, P = unknown sample, S = signal emitted by the antibody-analyte tracer complex (a,b), antibody tracer-analyte derivative complex (c,d) and from the sandwich complex (e,f). This and other schematic representations of an antibody do not take into account the existence of several binding sites (in the case of the IgG antibody: two).

antibodies of the same animal (in this case human) species (Fc portion). The latter presupposes, of course, that the antibodies present in the patient's blood, which are directed against completely different antigens, do not "capture" a significant fraction of the detection antibody. In practice such complications can be easily avoided by the choice of a corresponding high concentration of the detection antibody or by a so-called two-step performance of the assay. The separation of the sandwich complex and excess tracer, also necessary in this case prior to the measurement, is dealt with in Section 6.1.3.

The individual variations within these groups (cold and hot preincubation, one-step and two-step performance, double antibody method) as well as immunoassays which work without a tracer<sup>[1,2]</sup> cannot be dealt with here.

#### Definitions of Terms:

In scarcely any other area is there such a confusion of terms and abbreviations as in the field of immunoassays. The most well-known expression is the "radioimmunoassay" (RIA). Unfortunately it is often used for two different circumstances. First, it stands for competitive immunoassays with a radioactive analyte tracer, that is, for the assay type described in Group I, and second it is often employed as the generic term for all immunoassays with a radioactive label. The same can also be said for the enzyme immunoassay (EIA), fluorescence immunoassay (FIA), and luminescence or chemiluminescence immunoassay (LIA and CIA, respectively) which are distinguished from RIA only by the type of label used. A similar ambiguity exists with the acronym ELISA (enzyme-linked immunosorbent assay). This term is reserved by some authors for the excess reagent assay (Group III) with the enzyme label and by others is employed quite generally for all immunoassays with enzyme label.

Just as XIA (RIA, EIA, FIA, LIA, CIA) designates specifically assays of Group I, Group II does not have a generally accepted abbreviation. However, in this case one often comes across the expression SPALT (solid phase antigen luminescence technique). This describes an assay of Group II with luminogenic label, for which a particular but frequently used technique for separating excess antibody tracer is employed (see Section 6.1.3):

A synonym for sandwich assay (Group III) is the expression 2-site IXMA; this stands for "immuno-x... metric assay" (for example: IRMA: immunoradiometric assay; ILMA: immunoluminometric assay). The expression immunometric means that, in contrast to the competitive assays, one is dealing with an assay with excess reagent. Unfortunately the designation is also not uniform in this case. Hence, Group II, despite its competitive nature, is still designated with the expression "1-site IXMA". Strictly speaking the 1-site IXMA is, however, a very rare type of assay, which uses the same detection reagents as Group II (analyte derivative and antibody tracer), however, uses the antibody tracer in excess, and does not measure the complex formed from the analyte derivative and antibody tracer, but the complex formed from the analyte and antibody tracer.

#### 6.1.3. Separation Methods

As already mentioned the selective measurement of labeled immune complexes necessitate a prior separation of the unbound analyte tracer (in the case of Group I), of antibody tracer

not bound to the analyte derivative (Group II), or of unbound antibody tracer (Group III).

The first separation methods involved really difficult purification steps, for example chromatography or electrophoresis. Considerably more manageable, but today regarded as being antiquated, are the methods in which the immune complexes are precipitated by addition of salts or organic solvents, or the unbound analyte tracer is adsorbed on addition of activated charcoal or ion exchange resin. The requisite centrifugation step renders these methods as being no longer able to compete today.

Modern methods usually employ a solid phase. In the simplest case this is a small tube made of synthetic material, whose inner wall is coated with one of the detection reagents (coated tubes). At the same time these tubes serve as the reaction vessel for the immunological detection reaction. The separation immediately prior to measurement is reduced to just merely decanting off or removal of the reaction solution by suction (Fig. 4).

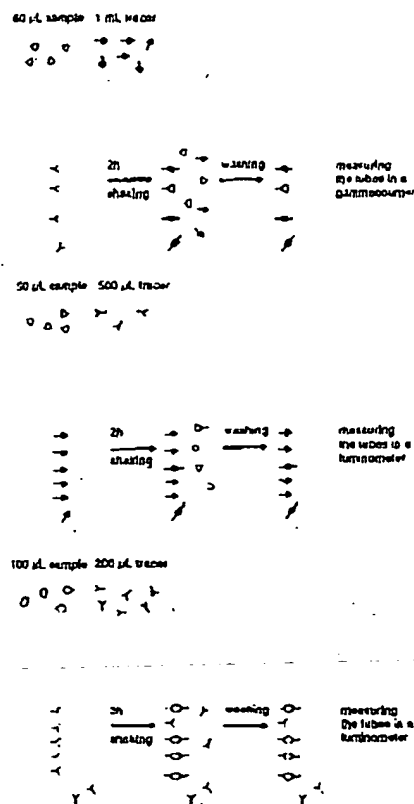


Fig. 4. Examples of commercial immunoassays performed with a coated tube: RIA-gnost T3 (top), a RIA and Berilux T3 (middle), a SPALT assay for determination of triiodothyronine (T3) and Berilux TSH (bottom), a 2-site ILMA for the determination of thyroid stimulating hormone (TSH) in serum.

The coating of the solid phase proceeds in the most simple case by direct adsorption of the detection reagent; the solid phase is kept in contact with a solution of the detection reagent for several hours. Under suitable conditions the adsorptive binding is so strong that the immobilized reagent cannot be dissolved by washing the solid phase. Binding can also result through an anchor protein, which adsorbs particularly well onto

the solid phase to which the detection reagent is covalently bonded by bifunctional reagents.<sup>[24]</sup> Often an antibody, which recognizes a structural element that is common to all antibodies of another animal species, is attached to the solid phase. For instance, antibodies which are directed against the Fc portion of mouse antibodies can be produced in rabbit. In this way antibodies can be anchored onto the solid phase which are less suitable for a direct adsorption. More of these universal solid phases are based on the high affinity binding between biotin and avidin (strept-avidin)<sup>[24]</sup> as well as between fluorescein and anti-fluorescein antibodies<sup>[184]</sup> (Fig. 5).

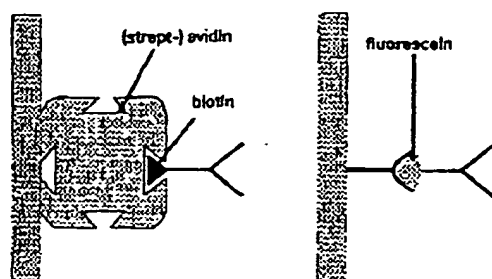


Fig. 5. The binding of a detection reagent (in this case an antibody) to a solid support can be achieved by means of the (strept-avidin/biotin system (left): The biotinylated antibody is supported by the (strept-)avidin adsorbed onto the solid support. A similar anchoring method is based on the strong bonding between fluorescein groups and antibodies directed against fluorescein (right).

Instead of coated tubes other coated solid phases can also be employed (synthetic spheres, magnetic particles, and membranes). These can also (cf. Fig. 4) participate directly in the immune reaction or merely have a separation function. In the latter case the immune reaction occurs in a homogeneous liquid phase (which has certain advantages with regards to the rate of reaction), and the separation function of the solid phase is only "switched on" once the reaction is completed (Fig. 6).

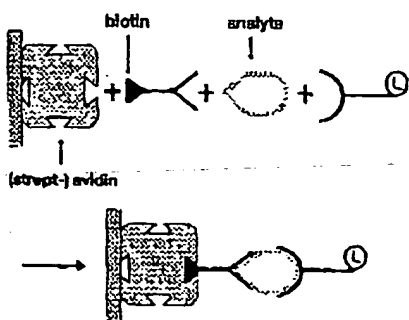


Fig. 6. After completion of the reaction between antibody labeled with either FITC or biotin, analyte, and analyte tracer in the liquid phase, the solid support coated with (strept-)avidin or anti-fluorescein antibodies, respectively, is added, and the sandwich complex is attached to the solid support.

With the OPUS system<sup>[113]</sup> separation is accomplished without further operation steps. The whole immune reaction takes place in a test module which is about the size of two sugar cubes. An essential component is a transparent polyester film, on the surface of which are three agarose layers. Complexes of the

antibody and fluorescent-labeled analyte derivative are embedded in the lower layer (1  $\mu\text{m}$  thick). The serum sample is applied to the top layer (10  $\mu\text{m}$  thick). The analyte molecules contained in the sample diffuse into the lower layer, where they—depending on their concentration—displace more or less tracer molecules from the antibody binding sites. Additional reagents such as aqueous solutions are not necessary here. Whereas antibodies are not able to leave the lower layer because of their size, the unbound tracer molecules are free to diffuse into the two upper layers. The antibody-tracer complexes which remain in the lowest layer are quantified by fluorescence detection. The light source and detector are situated below the test module. The middle agarose layer (10  $\mu\text{m}$  thick) acts as an optical filter due to its iron oxide content and prevents measurement of released tracer molecules which have diffused from the lower layer.

In the purely homogeneous assays there is no separation step because they are based on a changing signal in the formation of the immune complex. The first assay of this type was presented by Rubenstein and Ullman in 1971.<sup>[186a]</sup> This EMIT method (enzyme-modulated immunoassay technology) involves EIA; enzymatic activity of the enzyme label is inhibited by the binding of antibodies (Fig. 7). Another example of a homogeneous as-

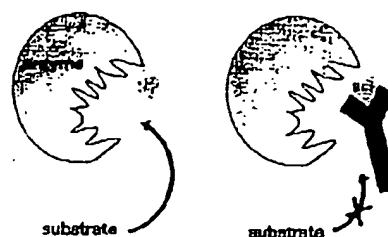


Fig. 7. In the EMIT method an antibody bound to the analyte tracer (analyte derivative labeled with the enzyme) prevents the catalytic conversion of the substrate (e.g. a chromogen) by the enzyme, for example, by steric hindrance at the active site.

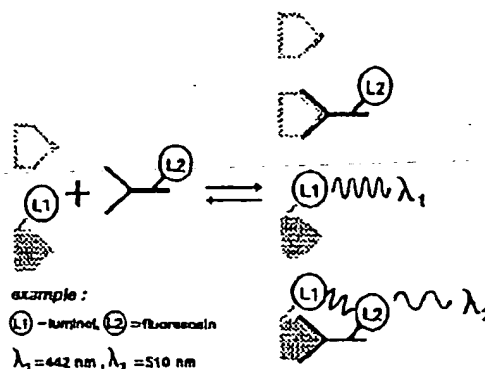


Fig. 8. Example of a homogeneous LIA: The energy transfer across the small distance between the two labels L1 and L2 results in an electronic excitation of L2. The intensity of the light emitted from L2 therefore correlates to the analyte concentration.

say which employs chemiluminescence energy transfer is shown in Figure 8. Disadvantages of these, at first glance, particularly elegant homogeneous assays are lower sensitivity of detection and a more pronounced susceptibility to interference.

## 6.2. Gene Probes

Whereas analytes in immunological determination methods are after all products of genetic information, nucleic acid sequences are the information itself. The qualitative and quantitative determination at this "primary level" is increasingly gaining significance.

The most important application at present is in the detection of pathogenic organisms (bacteria, viruses).<sup>(187)</sup> A particular advantage is that not only active viral infections, but also latent ones, are ascertainable by detection of the nucleic acid sequences. Thus, for example, infections with the AIDS virus can be detected already in the incubation phase of seronegative patients. In addition, the control of blood supplies for HIV, HTLV-I, Hepatitis B, etc. is much safer with the detection of the corresponding nucleic acid sequences than with an immunological test.

A series of hereditary diseases, such as diabetes mellitus, Lesch-Nyhan syndrome, phenylketonuria, and sickle cell anemia, can be detected reliably by tracing the mutated genes.<sup>(188)</sup> likewise the activation of different oncogenes which are involved in the formation of tumors.<sup>(189)</sup> In forensic medicine the detection of nucleic acid sequences is employed for solving cases involving sexual crimes or in tests for paternity suits.<sup>(190)</sup>

In contrast to immunoassays no antibodies are employed as direct detection reagents, but relatively short, mostly synthetic nucleic acid sequences (so-called gene probes) which are complementary to part of the analyte (so-called target sequence) (Fig. 9). These hybridize with the target sequence, that is, they

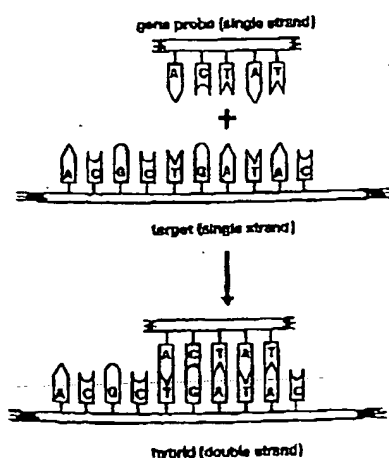


Fig. 9. The affinity of two nucleic acid strands arises from complementary base pairs: adenine (A) bonds to thymine (T) or uracil (U), and cytosine (C) to guanine (G). The gene probes employed are usually chains of 15–30 nucleotides.

bind with it to form a double strand which is held together by hydrogen bonding.

In the Southern-Blot method,<sup>(191)</sup> named after its founder, the DNA, on which presumably the sequence to be determined lies, is initially cut into defined fragments by restriction enzymes. These are separated by electrophoresis and the bands on the electrophoresis gel are transferred to a suitable carrier (e.g. nitrocellulose), whilst maintaining the relative positions of the bands. The individual DNA fragments (double-stranded) are denatured (that is, they are split into single strands) by heating,

in order to make them accessible to a hybridization with the labeled gene probe. One of the advantages of this method is that different probes can be employed simultaneously to trace several nucleic acid sequences.

The visualization of the fragments hybridized with the probes can be accomplished, for example, by applying a photographic film. The exposure times are greatly dependent on the label employed. For the  $^{32}\text{P}$  isotope used almost exclusively earlier, it was not unusual to have to wait for several weeks. By employing more modern luminogenic labels the exposure times can be drastically reduced. Methods that use fluorescent labels dispense with need for photographic film and, moreover, there is the advantage that different colored light signals can be received by employing different labels simultaneously. This can make the distinction of DNA fragments which exhibit similar electrophoresis profiles considerably easier.

In analogy to immunoassays there are also corresponding gene probe tests. The pendant of a 2-site IXMA<sup>(192)</sup> is shown in Figure 10. An analogue of the competitive immunoassay is the

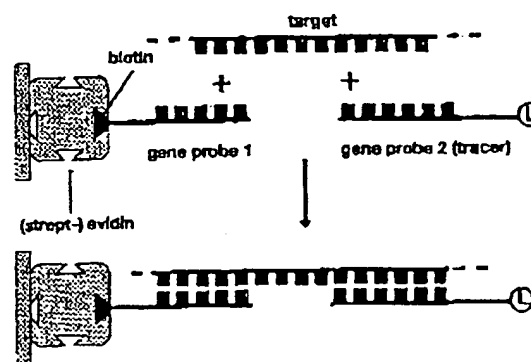


Fig. 10. The counterpart corresponding to the 2-site IXMA on gene probe site.

strand displacement assay<sup>(193)</sup> (Fig. 11). The only difference, in principle, to the XIA presented in Section 6.1.2. (Group I) is that the labeled gene probe (corresponds to the analyte tracer in XIA) and the target sequence (analyte) do not compete at the same time for the probe bound to the solid phase (corresponds to the antibody in XIA), but that the tracer gains a lead in time (principle of hot incubation; not an unusual experimental variable in XIA).

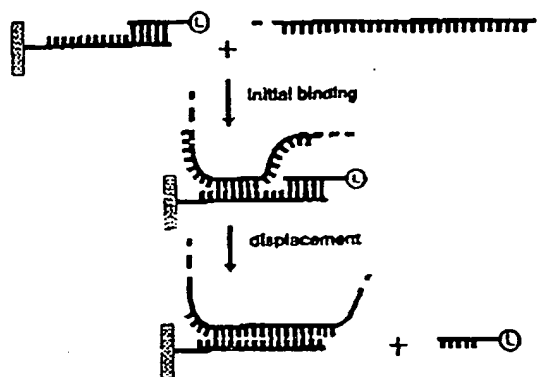


Fig. 11. Principle of the strand displacement assay.

The transfer of techniques from the field of immunoassays has also led to homogeneous gene probe test. In kissing probes (Fig. 12), two labeled probes bind to the target so closely to each other that an interaction is effected between the labels. Thus, only the changes in the signal occurring in this way can be viewed as highly specific.

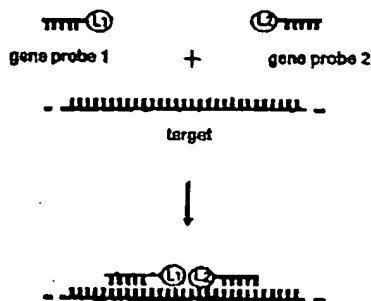


Fig. 12. Principle of the "kissing probes".

The acridinium label is able to intercalate double-stranded DNA and therefore is protected against attack from nucleophilic reagents. In this way it is possible to distinguish between single-stranded and double-stranded bound labels. This has been of use in the "hybridization protection assay".<sup>(194)</sup>

A revolutionary step for enhancing the sensitivity of gene probe tests was achieved by the PCR methods (polymerase chain reaction).<sup>(195)</sup> K. B. Mullis received the Nobel prize for the development of this method in 1993. The basic idea is original and at the same time simple. Whereas almost all attempts to improve the lower detection limit were directed at increasing the signal intensity and reducing the background signal, that is, to have the tracer in sight, with the PCR method, the target sequence is selectively replicated(!) and moreover quite simply. First, the target sequence present as a DNA double strand is cleaved into two single strands by denaturing with heat. The two single strands were then hybridized with complementary oligonucleotides and these were subsequently enzymatically

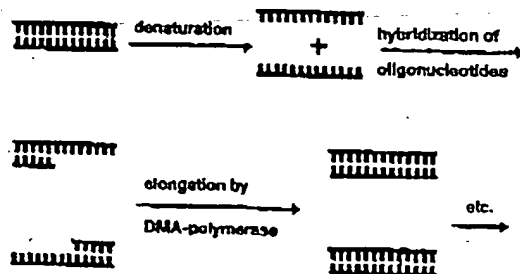


Fig. 13. PCR method.

elongated at the 3'-end with deoxynucleotide triphosphates. This process is repeated several times (Fig. 13) and after 20 cycles the target sequence is amplified by 100 000-fold.

## 7. Outlook

Even if a  $\gamma$  source which radiates continually for months to emit a signal often only required for a few seconds is everything but modern, there is much to be said, not only on ecological grounds, for the replacement of radioactive labels. With the labeling alternatives available today radioactive labels have, as far as detection sensitivity is concerned, been partly superseded. Why is it then that RIA did not die out long ago? A considerable advantage of RIA is tied up with one of its greatest disadvantages—as seen from an ecological standpoint—the emission of energy-rich radiation. In the thirty years of experience in the field of RIA this method has proved to be extraordinarily "robust". Thus, for example, in the case of the radioactive label the tracer is not influenced by its direct surroundings ("matrix effects") except for in the rarest of cases. Furthermore, the signal is not affected either in terms of its absolute value or in terms of its constancy by most external factors. In addition, the labeling of small analyte derivatives by an isotope inevitably results in fewer changes in properties compared to the introduction of a sterically demanding label. Thus, replacement of radioactive methods will understandably not occur overnight, but is more of a gradual process which is sustained by a steady increase in experience in dealing with nonradioactive labels and the synthesis of more effective labels. Since the most efficient luminescent labels and luminogenic enzyme substrates have only been available for a few years, one can expect an acceleration of this hitherto slow replacement of radioisotopes in the years to come.

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## EXHIBIT C

# PROTECTIVE GROUPS IN ORGANIC SYNTHESIS

THIRD EDITION

Theodora W. Greene  
The Rowland Institute for Science  
and

Peter G. M. Wuts  
Pharmacia and Upjohn Company



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# PREFACE TO THE THIRD EDITION

Organic synthesis has not yet matured to the point where protective groups are not needed for the synthesis of natural and unnatural products; thus, the development of new methods for functional group protection and deprotection continues. The new methods added to this edition come from both electronic searches and a manual examination of all the primary journals through the end of 1997. We have found that electronic searches of *Chemical Abstracts* fail to find many new methods that are developed during the course of a synthesis, and issues of selectivity are often not addressed. As with the second edition, we have attempted to highlight unusual and potentially useful examples of selectivity for both protection and deprotection. In some areas the methods listed may seem rather redundant, such as the numerous methods for THP protection and deprotection, but we have included them in an effort to be exhaustive in coverage. For comparison, the first edition of this book contains about 1500 references and 500 protective groups, the second edition introduces an additional 1500 references and 206 new protective groups, and the third edition adds 2349 new citations and 348 new protective groups.

Two new sections on the protection of phosphates and the alkyne-CH are included. All other sections of the book have been expanded, some more than others. The section on the protection of alcohols has increased substantially, reflecting the trend of the nineties to synthesize acetate- and propionate-derived natural products. An effort was made to include many more enzymatic methods of protection and deprotection. Most of these are associated with the protection of alcohols as esters and the protection of carboxylic acids. Here we have not attempted to be exhaustive, but hopefully, a sufficient number of cases are provided that illustrate the true power of this technology, so that the reader will examine some of the excellent monographs and review articles cited in the references. The Reactivity Charts in Chapter 10 are identical to those in the first edition. The chart number appears beside the name of each protective group when it is first introduced. No attempt was made to update these Charts, not only because of the sheer magnitude of the task, but because it is nearly impossible in

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## MISCELLANEOUS DERIVATIVES

### Ethoxycarbonyl: $\text{EtO}_2\text{C}-$

The ethoxycarbonyl group was developed for the protection of phosphonates. The derivative is prepared by reaction of tris(trimethylsilyl) phosphite with ethyl chloroformate and can be cleaved by hydrolysis of the ester followed by silylation with bis(trimethylsilyl)acetamide.<sup>1</sup>

### (Dimethylthiocarbamoyl)thio: $(\text{CH}_3)_2\text{NC}(\text{S})\text{S}-$

This group, used for internucleotide protection, is introduced with 8-quinoline-sulfonyl chloride,  $[(\text{CH}_3)_2\text{NC}(\text{S})\text{S}]_2$ , and  $\text{Ph}_3\text{P}$  and is cleaved with  $\text{BF}_3$  (dioxane,  $\text{H}_2\text{O}$ ,  $\pi$ ).<sup>2</sup>

1. M. Sekine, H. Mori, and T. Hata, *Bull. Chem. Soc. Jpn.*, **55**, 239 (1982).
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# REACTIVITIES, REAGENTS, AND REACTIVITY CHARTS

## REACTIVITIES

In the selection of a protective group, it is of paramount importance to know the reactivity of the resulting protected functionality toward various reagents and reaction conditions. The number of reagents available to the organic chemist is large; approximately 8000 reagents are reviewed in the excellent series of books by the Fiesers.<sup>1</sup> In an effort to assess the effect of a wide variety of standard types of reagents and reaction conditions on the different possible protected functionalities, 108 prototype reagents have been selected and grouped into 16 categories:<sup>2</sup>

- A. Aqueous
- B. Nonaqueous Bases
- C. Nonaqueous Nucleophiles
- D. Organometallic
- E. Catalytic Reduction
- F. Acidic Reduction
- G. Basic or Neutral Reduction
- H. Hydride Reduction
- I. Lewis Acids
- J. Soft Acids
- K. Radical Addition

- L. Oxidizing Agents  
 M. Thermal Reactions  
 N. Carbenoids  
 O. Miscellaneous  
 P. Electrophiles

These 108 reagents are used in the Reactivity Charts that have been prepared for each class of protective groups. The reagents and some of their properties are described on the following pages.

## REAGENTS

### A. AQUEOUS

1. pH < 1, 100°
2. pH < 1
3. pH 1
4. pH 2-4
5. pH 4-6
6. pH 6-8.5
7. pH 8.5-10
8. pH 10-12
9. pH > 12
10. pH > 12, 150°

#### Refluxing HBr

- 1 N HCl  
 0.1 N HCl  
 0.01 N HCl; 1-0.01 N HOAc  
 0.1 N H<sub>3</sub>BO<sub>3</sub>; phosphate buffer;  
 HOAc-NaOAc  
 H<sub>2</sub>O  
 0.1 N HCO<sub>3</sub><sup>-</sup>; 0.1 N OAc<sup>-</sup>; satd.  
 CaCO<sub>3</sub>  
 0.1 N CO<sub>3</sub><sup>2-</sup>; 1-0.01 N NH<sub>4</sub>OH;  
 0.01 N NaOH; satd Ca(OH)<sub>2</sub>  
 1-0.1 N NaOH

### B. NONAQUEOUS BASES

11. NaH
12. (C<sub>6</sub>H<sub>5</sub>)<sub>3</sub>CNa
13. [C<sub>10</sub>H<sub>8</sub>]<sup>-</sup> · Na<sup>+</sup>
14. CH<sub>3</sub>SOCH<sub>2</sub><sup>-</sup>Na<sup>+</sup>
15. KO-*i*-C<sub>4</sub>H<sub>9</sub>
16. LiN(*i*-C<sub>3</sub>H<sub>7</sub>)<sub>2</sub>
17. Pyridine; Et<sub>3</sub>N
18. NaNH<sub>2</sub>; NaNHR

- pK<sub>a</sub> = 32  
 pK<sub>a</sub> ≈ 37  
 pK<sub>a</sub> = 35  
 pK<sub>a</sub> = 19  
 (LDA) pK<sub>a</sub> = 36  
 pK<sub>a</sub> = 5; 10  
 pK<sub>a</sub> = 36

### C. NONAQUEOUS NUCLEOPHILES

19. NaOCH<sub>3</sub>/CH<sub>3</sub>OH, 25°
20. Enolate anion
21. NH<sub>3</sub>; RNH<sub>2</sub>; RNHOH

- pK<sub>a</sub> = 16  
 pK<sub>a</sub> = 20  
 pK<sub>a</sub> = 10

22. RS<sup>-</sup>; N<sub>3</sub><sup>-</sup>; SCN<sup>-</sup>
23. OAc<sup>-</sup>; X<sup>-</sup>
24. NaCH<sub>3</sub>, pH 12
25. HCN, cat. CN<sup>-</sup>, pH 6

pK<sub>a</sub> = 4.5

pK<sub>a</sub> = 9. For cyanohydrin formation

### D. ORGANOMETALLIC

26. RLi
27. RMgX
28. Organozinc
29. Organocopper
30. Wittig; ylide

Reformatsky reaction. Similar:

R<sub>2</sub>Cu; R<sub>2</sub>Cd

R<sub>2</sub>CuLi

Includes sulfur ylides

### E. CATALYTIC REDUCTION

31. H<sub>2</sub>/Raney Ni
32. H<sub>2</sub>/Pt, pH 2-4
33. H<sub>2</sub>/Pd-C
34. H<sub>2</sub>/Lindlar
35. H<sub>2</sub>/Rh-C or H<sub>2</sub>/Rh-Al<sub>2</sub>O<sub>3</sub>

Avoids hydrogenolysis of benzyl ethers

### F. ACIDIC REDUCTION

36. Zn/HCl
37. Zn/HOAc; SnCl<sub>2</sub>/HCl
38. Cr(II), pH 5

### G. BASIC OR NEUTRAL REDUCTION

39. Na//NH<sub>3</sub>
40. Al(Hg)
41. SnCl<sub>2</sub>/Py
42. H<sub>2</sub>S or HSO<sub>3</sub><sup>-</sup>

### H. HYDRIDE REDUCTION

43. LiAlH<sub>4</sub>
44. Li-*s*-Bu<sub>3</sub>BH, -50°
45. [(CH<sub>3</sub>)<sub>2</sub>CHCH(CH<sub>3</sub>)<sub>2</sub>]<sub>2</sub>BH
46. B<sub>2</sub>H<sub>6</sub>, 0°
47. NaBH<sub>4</sub>
48. Zn(BH<sub>4</sub>)<sub>2</sub>
49. NaBH<sub>3</sub>CN, pH 4-6
50. (*i*-C<sub>4</sub>H<sub>9</sub>)<sub>2</sub>AlH, -60°
51. Li(O-*i*-C<sub>4</sub>H<sub>9</sub>)<sub>2</sub>AlH, 0°

Li-Selectride  
 Disiamylborane

Neutral reduction

Dibal

## I. LEWIS ACIDS (ANHYDROUS CONDITIONS)

52.  $\text{AlCl}_3$ , 80°
53.  $\text{AlCl}_3$ , 25°
54.  $\text{SnCl}_4$ , 25°;  $\text{BF}_3 \cdot \text{Et}_2\text{O}$
55.  $\text{LiClO}_4$ ;  $\text{MgBr}_2$
56.  $\text{TsOH}$ , 80°
57.  $\text{TsOH}$ , 0°

For epoxide rearrangement  
Catalytic amount  
Catalytic amount

## J. SOFT ACIDS

58.  $\text{Hg}(\text{II})$
59.  $\text{Ag}(\text{I})$
60.  $\text{Cu}(\text{II})/\text{Py}$

For example, for Glaser coupling

## K. RADICAL ADDITION

61.  $\text{HBr}/\text{initiator}$
62.  $\text{HX}/\text{initiator}$
63.  $\text{NBS}/\text{CCl}_4$ ,  $h\nu$  or heat
64.  $\text{CHBr}_3$ ;  $\text{BrCCl}_3$ ;  $\text{CCl}_4/\text{In} \cdot$

"Acidic"  $\text{HX}$  addition; acidity  $\approx \text{TsOH}$ , 0°  
Neutral  $\text{HX}$  addition;  $\text{X} = \text{P}, \text{S}, \text{Se}, \text{Si}$   
Allylic bromination  
Carbon-halogen addition

## L. OXIDIZING AGENTS

65.  $\text{OsO}_4$
66.  $\text{KMnO}_4$ , 0°, pH 7
67.  $\text{O}_3$ , -50°
68.  $\text{RCO}_3\text{H}$ , 0°
69.  $\text{RCO}_3\text{H}$ , 50°
70.  $\text{CrO}_3/\text{Py}$
71.  $\text{CrO}_3$ , pH 1
72.  $\text{H}_2\text{O}_2/\text{OH}^-$ , pH 10-12
73. Quinone
74.  $^1\text{O}_2$
75.  $\text{CH}_3\text{SOCH}_3$ , 100°

Epoxidation of olefins; prototype for  
 $\text{H}_2\text{O}_2/\text{H}^+$   
Baeyer-Villiger oxidation of hindered  
ketones  
Collins oxidation  
Jones oxidation

Dehydrogenation  
Singlet oxygen  
( $\text{DMSO}$ );  $\text{HCO}_3^-$  may be added  
to maintain neutrality

Nonradical conditions

76.  $\text{NaOCl}$ , pH 10
77.  $\text{Aq. NBS}$
78.  $\text{I}_2$
79.  $\text{C}_6\text{H}_5\text{SeCl}$ ;  $\text{C}_6\text{H}_5\text{SeX}$
80.  $\text{Cl}_2$ ;  $\text{Br}_2$
81.  $\text{MnO}_2/\text{CH}_2\text{Cl}_2$
82.  $\text{NaIO}_4$ , pH 5-8
83.  $\text{SeO}_2$ , pH 2-4
84.  $\text{SeO}_2/\text{Py}$

In  $\text{EtOH}/\text{cat. Py}$

85.  $\text{K}_3\text{Fe}(\text{CN})_6$ , pH 7-10
86.  $\text{Pb}(\text{IV})$ , 25°
87.  $\text{Pb}(\text{IV})$ , 80°
88.  $\text{Ti}(\text{NO}_3)_3$ , pH 2

Phenol coupling  
Glycol and  $\alpha$ -hydroxy acid cleavage  
Oxidative decarboxylation  
Oxidative rearrangement of olefins

## M. THERMAL REACTIONS

89. 150°
90. 250°
91. 350°

Some Cope rearrangements and  
Cope eliminations  
Claisen or Cope rearrangement  
Ester cracking; Conia "ene" reaction

## N. CARBENOIDS

92.  $:\text{CCl}_2$
93.  $\text{N}_2\text{CHCO}_2\text{C}_2\text{H}_5/\text{Cu}$ , 80°
94.  $\text{CH}_2\text{I}_2/\text{Zn-Cu}$

Simmons-Smith addition

## O. MISCELLANEOUS

95.  $n\text{-Bu}_3\text{SnH}/\text{initiator}$
96.  $\text{Ni}(\text{CO})_4$
97.  $\text{CH}_3\text{N}_2$
98.  $\text{SOCl}_2$
99.  $\text{Ac}_2\text{O}$ , 25°
100.  $\text{Ac}_2\text{O}$ , 80°
101.  $\text{DCC}$
102.  $\text{CH}_3\text{I}$
103.  $(\text{CH}_3)_3\text{O}^+\text{BF}_4^-$
104. 1.  $\text{LiN-}i\text{-Pr}_2$ ; 2.  $\text{MeI}$
105. 1.  $\text{K}_2\text{CO}_3$ ; 2.  $\text{MeI}$

Acetylation  
Dehydration  
Dicyclohexylcarbodiimide,  
 $\text{C}_6\text{H}_{11}\text{N}=\text{C}=\text{NC}_6\text{H}_{11}$

Or  $\text{CH}_3\text{OSO}_2\text{F} = \text{Magic Methyl}$ ;  
**SEVERE POISON**  
For C-alkylation  
For O-alkylation

## P. ELECTROPHILES

106.  $\text{RCHO}$
107.  $\text{RCOCl}$
108.  $\text{C}^+\text{ion/olefin}$

For cation-olefin cyclization

## REACTIVITY CHARTS

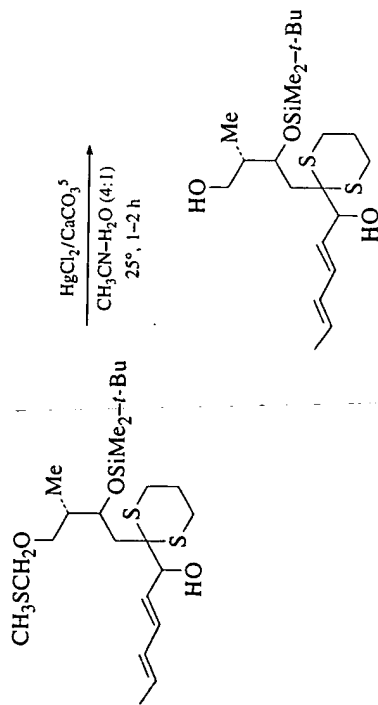
One requirement of a protective group is stability to a given reaction. The charts that follow were prepared as a guide to relative reactivities and thereby as an aid in the choice of a protective group. The reactivities in the charts were estimated

by the individual and collective efforts of a group of synthetic chemists. *It is important to realize that not all the reactivities in the charts have been determined experimentally and considerable conjecture has been exercised.* For those cases in which a literature reference was available concerning the use of a protective group and one of the 108 prototype reagents, the reactivity is printed in *italic type*. However, an exhaustive search for such references has not been made; therefore, the absence of *italic type* does not imply an experimentally unknown reactivity.

There are four levels of reactivity in the charts:

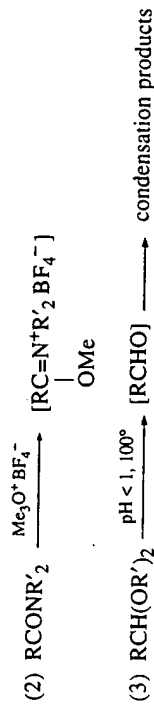
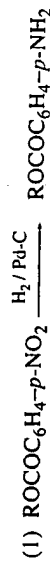
"H" (high) indicates that under the conditions of the prototype reagent, the protective group is readily removed to regenerate the original functional group.

"M" (marginal) indicates that the stability of the protected functionality is marginal and depends on the exact parameters of the reaction. The protective group may be stable, may be cleaved slowly, or may be unstable to the conditions. Relative rates are always important, as illustrated in the following example<sup>5</sup> (in which a monothioacetal is cleaved in the presence of a dithiane), and may have to be determined experimentally.



"L" (low) indicates that the protected functionality is stable under the reaction conditions.

"R" (reacts) indicates that the protected compound reacts readily, but that the original functional group is not restored. The protective group may be changed to a new protective group (eq. 1) or to a reactive intermediate (eq. 2), or the protective group may be unstable to the reaction conditions and react further (eq. 3).



The reactivities in the charts refer *only* to the protected functionality, not to atoms adjacent to the functional group; for example,  $\text{RCOOEt} \xrightarrow{\text{LDA}} \text{"L"}$  (low) reactivity of  $\text{PG(Et)}$ . However, if the protected functionality is  $\text{R}_2\text{CHCOOEt}$ , this substrate obviously *will* react with LDA. Reactivity of the entire substrate must be evaluated by the chemist.

Five reagents [#25:  $\text{HCN}$ , pH 6; #88:  $\text{Ti}(\text{NO}_3)_3$ ; #103:  $\text{Me}_3\text{O}^+\text{BF}_4^-$ ; #104:  $\text{LiN-}i\text{-Pr}_7/\text{MeI}$ ; and #105:  $\text{K}_2\text{CO}_3/\text{MeI}$ ] were added after some of the charts had been completed; reactivities to these reagents are not included for all charts.

The number used to designate a protective group (PG) in a Reactivity Chart is the same as that used in the body of the text in the *first* edition.

Protective group numbers in the Reactivity Charts are not continuous, since not all of the protective groups described in the text are included in the charts. The protective groups that are included in the Reactivity Charts are, in general, those that have been used most widely; consequently, considerable experimental information is available for them.

The Reactivity Charts were prepared in collaboration with the following chemists, to whom we are most grateful: John O. Albright, Dale L. Boger, Dr. Daniel J. Brunelle, Dr. David A. Clark, Dr. Jagabandhu Das, Herbert Estreicher, Anthony L. Fellu, Dr. Frank W. Hobbs, Jr., Paul B. Hopkins, Dr. Spencer Knapp, Dr. Pierre Lavallée, John Munroe, Jay W. Ponder, Marcus A. Tius, Dr. David R. Williams, and Robert E. Wolf, Jr.

<sup>1</sup> L. F. Fieser and M. Fieser, *Reagents for Organic Synthesis*, Wiley-Interscience, New York, 1967, Vol. 1; M. Fieser and L. F. Fieser, Vols. 2-7, 1969-1979; M. Fieser, Vols. 8-17, 1980-1994.

<sup>2</sup> The categories and prototype reagents used in this study are an expansion of an earlier set of 11 categories and 60 prototype reagents,<sup>3</sup> originally compiled for use in LHASA<sup>4</sup> (Logic and Heuristics Applied to Synthetic Analysis), a long-term research program at Harvard University for Computer-Assisted Synthetic Analysis.

<sup>3</sup> E. J. Corey, H. W. Orf, and D. A. Pensak, *J. Am. Chem. Soc.*, **98**, 210 (1976).

<sup>4</sup> Selected references include E. J. Corey, *Quart. Rev., Chem. Soc.*, **25**, 455 (1971); H. W. Orf, Ph. D. Thesis, Harvard University, 1976.

<sup>5</sup> E. J. Corey and M. G. Bock, *Tetrahedron Lett.*, 2643 (1975).





**Reactivity Chart 1. Protection for the Hydroxyl Group: Ethers (Continued)**

		Na/NH <sub>3</sub>										Al (Hg)										SnCl <sub>2</sub> /Py										HNO <sub>3</sub> /H <sub>2</sub> S										LiAlH <sub>4</sub>										Li <sup>+</sup> -Bu <sub>3</sub> BH										(C <sub>2</sub> H <sub>5</sub> ) <sub>3</sub> Li										B <sub>2</sub> H <sub>6</sub> , 0°										NaBH <sub>4</sub>										Zn(Bu <sub>4</sub> ) <sub>2</sub>										NaBH <sub>3</sub> CN pH 4-6										Li <sup>+</sup> -Bu <sub>3</sub> AlH										Li <sup>+</sup> (OC <sub>2</sub> H <sub>5</sub> ) <sub>3</sub> AlH										AlCl <sub>3</sub> , 80°										AlCl <sub>3</sub> , 25°										SnCl <sub>4</sub> , BF <sub>3</sub>										LiClO <sub>4</sub> , MgBr <sub>2</sub>										TeOH, 80°										Ti <sub>2</sub> O <sub>3</sub> , 0°										Hg (II)										Ag (I)										Cu (II)/Py										HBr/Zn										HBr/In										NBS/CCl <sub>4</sub>										Br <sub>2</sub> /CCl <sub>4</sub> /Zn										O <sub>2</sub> O <sub>4</sub>										HNO <sub>3</sub> , pH 7, 0°										O <sub>3</sub> , -50°										RCO <sub>3</sub> H, 0°										RCO <sub>3</sub> H, 50°										CrO <sub>3</sub> /Py										CrO <sub>3</sub> , pH 1										H <sub>2</sub> O <sub>2</sub> , pH 10-12										Quinone										I <sub>2</sub>										DMSO, 100°										NaOCl, pH 10										aq NBS																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
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**Reactivity Chart 1. Protection for the Hydroxyl Group: Ethers (Continued)**

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### Reactivity Chart 2. Protection for Hydroxyl Group: Esters

1. Formate Ester
3. Acetate Ester
6. Trichloroacetate Ester
10. Phenoxycetate Ester
19. Isobutyrate Ester
22. Pivaloate Ester
23. Adamantoate Ester
27. Benzoate Ester
31. 2,4,6-Trimethylbenzoate (Mesitoate) Ester
34. Methyl Carbonate
36. 2,2,2-Trichloroethyl Carbonate
39. Allyl Carbonate
41. *p*-Nitrophenyl Carbonate
42. Benzyl Carbonate
46. *p*-Nitrobenzyl Carbonate
47. *S*-Benzyl Thiocarbonate
48. *N*-Phenylcarbamate
51. Nitrate Ester
53. 2,4-Dinitrophenylsulfonate Ester

(See chart, pp. 713-715.)

Reactivity Chart 2. Protection for the Hydroxyl Group: Esters

PG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38			
	A. AQUEOUS										B. BASIC										C. NUCLEOPHILIC										D. ORGANOMET.										
	pH<1, 100°										NaH										RLi										RMgX										
	pH<1										(C <sub>10</sub> H <sub>8</sub> ) <sup>-</sup> Na <sup>+</sup>										Enolate										Organozinc										
	pH 1										MeSOCH <sub>2</sub> <sup>-</sup> Na <sup>+</sup>										RS <sup>-</sup> , N <sub>3</sub> <sup>-</sup> , SCN <sup>-</sup>										Organocopper										
	pH 2-4										KO <sup>-</sup> t-Bu										OAc <sup>-</sup> , X <sup>-</sup>										Wittig ylide										
	pH 4-6										LAN-1-Pr <sub>2</sub>										NACN, pH 12										H <sub>2</sub> /Raney (Ni)										
	pH 6-8.5										Py; R <sub>3</sub> N										HCN, pH 6										H <sub>2</sub> /Pt pH 2-4										
	pH 8.5-10										NaNH <sub>2</sub>																				H <sub>2</sub> /Pd										
	pH 10-12																														H <sub>2</sub> /Lindlar										
	pH>12																														Zn/Rh										
	pH>12, 150°																														Zn/HCl										
																															Zn/HOAc										
																															C <sub>6</sub> (H <sub>5</sub> ) <sub>5</sub> pH 5										

Reactivity Chart 2. Protection for the Hydroxyl Group: Esters (Continued)

PG	Na/NH <sub>3</sub> Al(Hg) SnCl <sub>2</sub> /Py HSO <sub>3</sub> <sup>-</sup> ; H <sub>2</sub> S				LiAlH <sub>4</sub> Li <sup>-</sup> -g-Bu <sub>3</sub> BH (C <sub>5</sub> H <sub>11</sub> ) <sub>2</sub> BH B <sub>2</sub> H <sub>6</sub> , 0° NaBH <sub>4</sub> Zn(BH <sub>4</sub> ) <sub>2</sub> NaBH <sub>3</sub> CN pH 4-6 i-Bu <sub>2</sub> AlH Li(O <sup>-</sup> tBu) <sub>3</sub> AlH							AlCl <sub>3</sub> , 80° AlCl <sub>3</sub> , 25° SnCl <sub>4</sub> ; BF <sub>3</sub> LiClO <sub>4</sub> ; MgBr <sub>2</sub> TsOH, 80° TsOH, 0°					Hg(II) Ag(I) Cu(II)/Py			HBr/Zn <sup>+</sup> HX/In <sup>+</sup> NBS/CCl <sub>4</sub> Br <sub>2</sub> CCl/In <sup>+</sup>			OsO <sub>4</sub> KMnO <sub>4</sub> , pH 7, 0° O <sub>3</sub> , -50° RCO <sub>3</sub> H, 0° RCO <sub>3</sub> H, 50° CrO <sub>3</sub> /Py CrO <sub>3</sub> , pH 1 H <sub>2</sub> O <sub>2</sub> pH 10-12 Quinone I <sub>2</sub> O <sub>2</sub> DMSO, 100° NaOCl pH 10 aq NBS																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																			
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1	H	L	L	L	H	H	M	M	M	M	M	H	M	H	H	L	L	H	L	L	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L

Reactivity Chart 2. Protection for the Hydroxyl Group: Esters (Continued)

	I <sub>2</sub> PhSeX; PhSeCl Br <sub>2</sub> ; Cl <sub>2</sub> MnO <sub>2</sub> /CH <sub>2</sub> Cl <sub>2</sub> NaIO <sub>4</sub> pH 5-8 SeO <sub>2</sub> pH 2-4 SeO <sub>2</sub> /Py K <sub>3</sub> Fe(CN) <sub>6</sub> , pH 8 Pb(IV), 25° Pb(IV), 80° Ti(NO <sub>3</sub> ) <sub>3</sub>												150° 250° 350°			:CCl <sub>2</sub> N <sub>2</sub> CHCO <sub>2</sub> R/Cu CH <sub>2</sub> I <sub>2</sub> /Zn(Cu)			R <sub>3</sub> SnH/In. Ni(CO) <sub>4</sub> CH <sub>2</sub> N <sub>2</sub> SOCl <sub>2</sub> Ac <sub>2</sub> O, 25° Ac <sub>2</sub> O, 80° DCC MeI Me <sub>3</sub> O <sup>+</sup> BF <sub>4</sub> <sup>-</sup> 1. LDA 2. MeI 1. K <sub>2</sub> CO <sub>3</sub> 2. MeI												RCHO RCOCl C <sup>+</sup> /olefin																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
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1	L	L	L	L	L	M	M	M	L	L		L	M	H	L	L	L	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L

**Reactivity Chart 3. Protection for 1,2- and 1,3-Diols**

1. Methylenedioxy Derivative
2. Ethylidene Acetal
6. Acetonide Derivative
11. Benzylidene Acetal
13. *p*-Methoxybenzylidene Acetal
18. Methoxymethylene Acetal
20. Dimethoxymethylenedioxy Derivative
28. Cyclic Carbonates
29. Cyclic Boronates

(See chart, pp. 717-719.)

Reactivity Chart 3. Protection for 1,2- and 1,3-Diols

PG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38		
	pH<1, 100°										pH<1										Enolate										RMgX									
	pH 1										(C <sub>10</sub> H <sub>8</sub> ) <sup>-</sup> Na <sup>+</sup>										NH <sub>3</sub> ; RNH <sub>2</sub>										Organozinc									
	pH 2-4										MesOCH <sub>2</sub> -Na <sup>+</sup>										RS <sup>-</sup> ; N <sub>3</sub> <sup>-</sup> ; SCN <sup>-</sup>										Organocopper									
	pH 4-6										KO <sup>-</sup> t-Bu										LiN-1-Pr <sub>2</sub>										Wittig; ylide									
	pH 6-8.5										Py; R <sub>3</sub> N										NaNH <sub>2</sub>										H <sub>2</sub> /Raney (Ni)									
	pH 8.5-10										LiN-1-Pr <sub>2</sub>										NaOMe										H <sub>2</sub> /Pt pH 2-4									
	pH 10-12										LiN-1-Pr <sub>2</sub>										NaOMe										H <sub>2</sub> /Pd									
	pH>12										LiN-1-Pr <sub>2</sub>										NaOMe										H <sub>2</sub> /Rh									
	pH>12, 150°										LiN-1-Pr <sub>2</sub>										NaOMe										Zn/HCl									
	pH>12, 150°										LiN-1-Pr <sub>2</sub>										NaOMe										Zn/HOAc									
	pH>12, 150°										LiN-1-Pr <sub>2</sub>										NaOMe										Cr(II), pH 5									

Reactivity Chart 3. Protection for 1,2- and 1,3-Diols (Continued)

PG	Na/NH <sub>3</sub> Al (Hg) SnCl <sub>2</sub> /Py HSO <sub>3</sub> <sup>-</sup> , H <sub>2</sub> S				LiAlH <sub>4</sub> Li <sup>-</sup> -Bu <sub>3</sub> BH (C <sub>5</sub> H <sub>11</sub> ) <sub>2</sub> BH B <sub>2</sub> H <sub>6</sub> , 0° NaBH <sub>4</sub> Zn (BH <sub>4</sub> ) <sub>2</sub> NaBH <sub>3</sub> CN pH 4-6 1-Bu <sub>2</sub> AlH Li (O <sup>-</sup> Bu) <sub>3</sub> AlH							AlCl <sub>3</sub> , 80° AlCl <sub>3</sub> , 25° SnCl <sub>4</sub> ; BF <sub>3</sub> LiClO <sub>4</sub> ; MgBr <sub>2</sub> TsOH, 80° TsOH, 0°					Hg(II) Ag(I) Cu(II)/Py			HBz/In. HX/In. NBS/CCl <sub>4</sub> Br <sub>2</sub> CCl/In.				OxO <sub>4</sub> KMnO <sub>4</sub> , pH 7, 0° O <sub>3</sub> , -50° RCO <sub>3</sub> H, 0° RCO <sub>3</sub> H, 50° CrO <sub>3</sub> /Py CrO <sub>3</sub> , pH 1 H <sub>2</sub> O <sub>2</sub> pH 10-12 Quinone I <sub>2</sub> DMSO, 100° NaOCl pH 10 aq NBS																	
39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77			
G.				H. HYDRIDE REDN.										I.					J.				K.				L. OXIDANTS														
1	L	L	L	L	L	L	L	L	L	L	L	L	H	H	H	L	M	L	L	L	L	R	L	L	L	L	L	L	R	L	L	L	L	L	L	L	L	L			
2	L	L	L	L	L	L	L	L	L	L	L	L	H	H	H	L	M	L	L	L	L	M	L	L	L	L	L	L	R	L	L	L	L	L	L	L	L	L			
6	L	L	L	L	L	L	L	L	L	L	L	L	H	H	H	L	M	L	L	L	L	M	L	L	L	L	L	L	R	L	L	L	L	L	L	L	L	L			
11	H	L	L	L	L	L	L	L	L	L	L	L	H	H	H	L	M	L	L	L	L	H	L	L	L	L	L	L	R	L	L	L	L	L	L	L	L	L			
12	H	L	L	L	L	L	L	L	L	L	L	L	H	H	H	L	M	L	L	L	L	H	L	R	L	L	L	L	R	L	H	L	L	L	L	L	L	M			
18	L	L	L	L	R	L	L	L	L	L	M	L	H	H	H	M	R	H	L	L	L	H	L	H	L	L	L	M	L	H	H	L	H	L	L	L	L	M			
20	L	L	L	L	R	L	L	L	L	L	M	L	H	H	H	M	R	H	L	L	L	H	L	H	L	L	L	M	L	H	H	L	H	L	L	L	M				
28	H	L	L	L	H	L	L	L	L	L	H	L	H	H	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M				
29	H	M	L	L	H	H	H	H	H	H	H	H	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	H	H				

Reactivity Chart 3. Protection for 1,2- and 1,3-Diols (Continued)

PG	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108
	L. OXIDANTS												M.			N.			O. MISCELLANEOUS										P.		
1	L	L	L	L	L	L	L	L	L	L	L	L	L	M	L	L	L	L	L	L	L	M	L	L	L	M	L	L	L	L	H
2	L	L	L	L	L	L	M	L	L	L	M	L	L	M	L	L	L	L	L	L	L	M	L	L	L	M	L	L	L	L	H
6	L	L	L	L	L	L	M	L	L	L	M	L	L	M	L	L	L	L	L	L	L	M	L	L	L	M	L	L	L	L	H
11	L	L	R	L	L	H	L	L	L	L	M	L	M	H	L	L	L	L	L	L	L	M	L	L	L	M	L	L	L	L	H
12	L	L	R	L	L	H	L	L	L	L	M	L	M	H	L	M	L	L	L	L	L	M	L	L	L	M	L	L	L	L	H
18	L	L	L	L	M	H	L	L	L	L	H	L	M	H	L	L	L	L	L	L	L	M	R	L	L	L	R	L	L	L	H
20	L	L	L	L	M	R	L	L	L	L	H	L	M	H	L	L	L	L	L	L	L	M	R	L	L	L	R	L	L	L	H
28	L	L	L	L	L	L	L	L	L	L	L	L	M	H	L	L	L	L	L	L	L	M	R	L	L	L	L	L	L	L	L
29	L	L	L	L	H	H	L	H	H	H	H	L	H	H	L	L	L	M	L	L	M	H	L	L	L	R	L	L	L	M	H

1. Methyl Ether
2. Methoxymethyl Ether
3. 2-Methoxyethoxymethyl Ether
4. Methylthiomethyl Ether
6. Phenacyl Ether
7. Allyl Ether
8. Cyclohexyl Ether
9. *t*-Butyl Ether
10. Benzyl Ether
11. *o*-Nitrobenzyl Ether
12. 9-Anthrylmethyl Ether
13. 4-Picolyl Ether
15. *t*-Butyldimethylsilyl Ether
16. Aryl Acetate
17. Aryl Pivaloate
18. Aryl Benzoate
19. Aryl 9-Fluorene-9-carboxylate
20. Aryl Methyl Carbonate
21. Aryl 2,2,2-Trichloroethyl Carbonate
22. Aryl Vinyl Carbonate
23. Aryl Benzyl Carbonate
25. Aryl Methanesulfonate

Catechols

27. Methyleneedioxy Derivative
28. Acetonide Derivative
30. Diphenylmethylenedioxy Derivative
31. Cyclic Borates
32. Cyclic Carbonates

(See chart, pp. 721–723.)

[illegible]

**Reactivity Chart 4. Protection for Phenols and Catechols (Continued)**

PG	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77				
	Na/NH <sub>3</sub>	Al(Hg)	SnCl <sub>2</sub> /Py	H <sub>2</sub> S	LiAlH <sub>4</sub>	Li-2-Bu <sub>3</sub> BH	(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub> BH	B <sub>2</sub> H <sub>6</sub> , 0°	NaBH <sub>4</sub>	Zn(BH <sub>4</sub> ) <sub>2</sub>	NaBH <sub>3</sub> CN pH 4-6	i-Bu <sub>3</sub> AlH	Li(O <sub>2</sub> Cu) <sub>3</sub> AlH	AlCl <sub>3</sub> , 80°	AlCl <sub>3</sub> , 25°	SnCl <sub>4</sub> , BF <sub>3</sub>	LiClO <sub>4</sub> , MgBr <sub>2</sub>	TeOH, 80°	TeOH, 0°	Hg(II)	Ag(I)	Cu(II)/Py	HBr/Zn	HX/Zn	NBS/CCl <sub>4</sub>	BF <sub>3</sub> CCl <sub>3</sub> /Zn	CoO <sub>4</sub>	K <sub>2</sub> MoO <sub>4</sub> pH 7, 0°	O <sub>3</sub> , -50°	RCO <sub>3</sub> H, 0°	RCO <sub>3</sub> H, 50°	CrO <sub>3</sub> /Py	CrO <sub>3</sub> , pH 1	H <sub>2</sub> O <sub>2</sub> pH 10-12	Quinone	l <sub>2</sub> O <sub>2</sub>	DMSO, 100°	NaOCl pH 10	aq NBS				
	G.					H. HYDRIDE	REDN.													J.			K.																				
1	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L		
2	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
3	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
4	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
6	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
7	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
8	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
9	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
10	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
11	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
12	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
13	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
15	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
16	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
17	R	L	L	L	L	L	L	L	L	L	L	L	L	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
18	R	L	L	L	L	L	L	L	L	L	L	L	L	R	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
19	R	L	L	L	L	L	L	L	L	L	L	L	L	R	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
20	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
21	R	M	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
22	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
23	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
25	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
27	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
28	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
30	R	M	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
31	R	M	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
32	R	L	L	L	L	L	L	L	L	L	L	L	L	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L

**Reactivity Chart 4. Protection for Phenols and Catechols (Continued)**

[illegible]



## Reactivity Chart 5. Protection for the Carbonyl Group

1. Dimethyl Acetals and Ketals
3. Bis(2,2,2-trichloroethyl) Acetals and Ketals
5. 1,3-Dioxanes
6. 5-Methylene-1,3-dioxanes
7. 5,5-Dibromo-1,3-dioxanes
8. 1,3-Dioxolanes
9. 4-Bromomethyl-1,3-dioxolanes
10. 4-*o*-Nitrophenyl-1,3-dioxolanes
11. *S,S'*-Dimethyl Acetals and Ketals
19. 1,3-Dithianes
20. 1,3-Dithiolanes
24. 1,3-Oxathiolanes
26. *O*-Trimethylsilyl Cyanohydrins
29. *N,N*-Dimethylhydrazones
30. 2,4-Dinitrophenylhydrazones
33. *O*-Phenylthiomethyl Oximes
34. Substituted Methylene Derivatives
43. Bismethylenedioxy Derivatives

(See chart, pp. 725-727.)

Reactivity Chart 5. Protection for the Carbonyl Group

	pH<1, 100°										pH>12, 150°																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
	pH<1										pH>12, 150°										NaH								Ph <sub>3</sub> CNa								NaOMe								R <sub>2</sub> Li										R <sub>2</sub> MgX										Organozinc										Organocopper										Wittig; ylide										H <sub>2</sub> /Raney (Ni)										H <sub>2</sub> /Pt pH 2-4										H <sub>2</sub> /Pd										H <sub>2</sub> /Lindlar										H <sub>2</sub> /Rh										Zn/HCl										Zn/HOAc										Cr (II), pH 5																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
PG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
	A. AQUEOUS										B. BASIC								C. NUCLEOPHILIC								D. ORGANOMET.										E. CAT. REDN.										F.																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																													
	H	H	H	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L

Reactivity Chart 5. Protection for the Carbonyl Group (Continued)

[illegible]

### Reactivity Chart 5. Protection for the Carbonyl Group (Continued)

Activity Chart of 1. Protobacter for the Safety Group (Continued)																																					
PG	I <sub>2</sub> PhSeX; PhSCl Br <sub>2</sub> ; Cl <sub>2</sub> MnO <sub>2</sub> /CH <sub>2</sub> Cl <sub>2</sub> NaIO <sub>4</sub> pH 5-8 SeO <sub>2</sub> pH 2-4 SeO <sub>2</sub> /Py K <sub>3</sub> Fe(CN) <sub>6</sub> , pH 8 Pb(IV), 25° Pb(IV), 80° Tl(NO <sub>3</sub> ) <sub>3</sub>										150° 250° 350°			tCCl <sub>2</sub> N <sub>2</sub> CHCO <sub>2</sub> R/Cu CH <sub>2</sub> I <sub>2</sub> /Zn(Cu)			R <sub>3</sub> SnH/In· Ni(CO) <sub>4</sub> CH <sub>2</sub> N <sub>2</sub> SOCl <sub>2</sub> Ac <sub>2</sub> O, 25° Ac <sub>2</sub> O, 80° DCC MeI Me <sub>3</sub> O <sup>+</sup> BF <sub>4</sub> 1. LDA 2. MeI 1. K <sub>2</sub> CO <sub>3</sub> 2. MeI			RCHO RCOCl C <sup>+</sup> /olefin																	
	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108						
O. MISCELLANEOUS																																					
P.																																					
1	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L				
3	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L				
5	L	L	R	L	L	M	L	L	L	L	L	L	L	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L				
6	R	R	R	L	R	M	R	L	R	R	L	L	L	R	R	R	R	L	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L				
7	L	L	R	L	L	M	L	L	L	L	L	L	L	R	L	M	H	R	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L				
8	L	L	R	L	L	H	L	L	L	L	L	L	L	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L				
9	L	L	R	L	M	H	L	L	L	L	L	L	L	R	L	L	H	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L				
10	L	L	R	L	M	H	L	L	L	L	L	L	L	R	L	L	L	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L				
11	H	L	R	M	R	M	L	L	R	R	L	L	L	R	M	M	L	M	L	L	L	L	L	L	L	L	R	R	L	L	L	L	L				
19	L	L	R	M	R	M	L	L	L	M	L	L	L	R	M	M	L	M	L	L	L	L	L	L	L	H	L	L	L	L	L	L	L				
20	H	L	R	M	R	M	L	L	R	R	L	L	R	M	M	L	M	L	L	L	L	L	L	L	H	R	R	L	L	L	L	L	L				
24	L	L	R	L	R	M	L	L	R	R	L	L	R	M	M	L	M	L	L	L	L	R	M	L	R	R	R	L	L	L	L	L	L	L			
26	L	L	R	R	R	R	L	R	R	R	L	M	R	R	L	R	L	L	L	L	L	R	L	L	L	M	R	L	L	L	L	L	L	L			
29	L	L	H	M	H	L	M	L	R	R	L	R	R	R	R	R	R	L	L	L	L	L	L	L	R	R	R	L	L	L	L	L	L	L			
30	L	L	L	M	H	L	L	L	R	R	L	M	R	R	R	R	R	L	L	L	L	L	L	L	M	R	R	L	L	L	L	L	L	L			
33	L	L	R	L	R	M	L	L	R	R	L	R	R	R	R	R	M	L	L	L	L	L	L	L	R	R	R	L	L	L	L	L	L	L			
34	L	L	R	R	R	M	L	L	L	L	R	L	M	R	R	R	R	L	L	L	L	L	L	L	L	M	M	L	L	L	L	L	L	L			
43	L	L	L	L	L	L	L	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	M	L	L	M	M	L	L	L	L	L	L	L			

# Reactivity Chart 6. Protection for the Carboxyl Group

## Esters

1. Methyl Ester
2. Methoxymethyl Ester
3. Methylthiomethyl Ester
4. Tetrahydropyranyl Ester
7. Benzoyloxymethyl Ester
8. Phenacyl Ester
13. *N*-Phthalimidomethyl Ester
15. 2,2,2-Trichloroethyl Ester
16. 2-Haloethyl Ester
21. 2-(*p*-Toluenesulfonyl)ethyl Ester
23. *t*-Butyl Ester
27. Cinnamyl Ester
30. Benzyl Ester
31. Triphenylmethyl Ester
33. Bis(*o*-nitrophenyl)methyl Ester
34. 9-Anthrylmethyl Ester
35. 2-(9,10-Dioxo)anthrylmethyl Ester
42. Piperonyl Ester
45. Trimethylsilyl Ester
47. *t*-Butyldimethylsilyl Ester
50. *S*-*t*-Butyl Ester
59. 2-Alkyl-1,3-oxazolines

## Amides and Hydrazides

64. *N,N*-Dimethylamide
68. *N*-7-Nitroindolylamide
71. Hydrazides
72. *N*-Phenylhydrazide
73. *N,N'*-Diisopropylhydrazide

(See chart, pp. 729-731.)

Reactivity Chart 6. Protection for the Carboxyl Group

PC	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
	Aqueous										B, BASIC										C, NUCLEOPHILIC										D, ORGANOMET.								RLi		RMgX		Organosilic		Organocopper		Wittig, ylide		H <sub>2</sub> /Raney (Ni)		H <sub>2</sub> /Pt, pH 2-4		H <sub>2</sub> /Pd		H <sub>2</sub> /Lindlar		H <sub>2</sub> /Rb		Zn/HCl		Zn/HOAc		Cr(III), pH 5																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																														
	pH<1, 100°	pH<1	pH 1	pH 2-4	pH 4-6	pH 6-8.5	pH 8.5-10	pH 10-12	pH>12	pH>12, 150°	NaN	Ph <sub>3</sub> CNa	(C <sub>10</sub> H <sub>8</sub> ) <sup>+</sup> Na <sup>+</sup>	MeSOCH <sub>2</sub> <sup>+</sup> Na <sup>+</sup>	KO-t-Bu	LiN-1-Pr <sub>2</sub>	Py, R <sub>3</sub> N	NmH <sub>2</sub>	NaOMe	Epoxide	NH <sub>3</sub> , RNH <sub>2</sub>	RS <sup>-</sup> , N <sub>3</sub> <sup>-</sup> , SCN <sup>-</sup>	OAc <sup>-</sup> , X <sup>-</sup>	NaCN, pH 12	HCN, pH 6	RLi	RMgX	Organosilic	Organocopper	Wittig, ylide	H <sub>2</sub> /Raney (Ni)	H <sub>2</sub> /Pt, pH 2-4	H <sub>2</sub> /Pd	H <sub>2</sub> /Lindlar	H <sub>2</sub> /Rb	Zn/HCl	Zn/HOAc	Cr(III), pH 5																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H

**Reactivity Chart 6. Protection for the Carboxyl Group (Continued)**

	Na/NH <sub>3</sub>	Al(Hg)	SnCl <sub>2</sub> /Py	HSO <sub>3</sub> ·H <sub>2</sub> S	LiAlH <sub>4</sub>	Li <sup>-</sup> -Bu <sub>3</sub> BH	(C <sub>5</sub> H <sub>11</sub> ) <sub>2</sub> BH	B <sub>2</sub> H <sub>6</sub> ·0°	Zn(BH <sub>4</sub> ) <sub>2</sub>	NaBH <sub>3</sub> CN pH 4-6	Li <sup>-</sup> -Bu <sub>2</sub> AlH	Li(O <sub>2</sub> Bu) <sub>3</sub> AlH	AlCl <sub>3</sub> ·80°	AlCl <sub>3</sub> ·25°	SnCl <sub>4</sub> ·BF <sub>3</sub>	LiClO <sub>4</sub> ·MgBr <sub>2</sub>	TiCl <sub>4</sub> ·80°	TiCl <sub>4</sub> ·0°	Hg(II)	Ag(I)	Cu(II)/Py	NBr/In·	HX/In·	NBS/CCl <sub>4</sub>	Br <sub>3</sub> CCl/In·	O <sub>2</sub>	KNO <sub>3</sub> ·pH 7, 0°	O <sub>3</sub> ·-50°	RCO <sub>3</sub> H·0°	RCO <sub>3</sub> H·50°	CrO <sub>3</sub> /Py	CrO <sub>3</sub> ·pH 1	H <sub>2</sub> O <sub>2</sub> ·pH 10-12	Quinone	IO <sub>2</sub>	DMSO, 100°	NaOCl pH 10	eq NBS			
PG	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77		
	G.				H.				HYDRIDE	REDN.									J.			K.																			
1	R	L	L	L	R	M	L	L	L	L	R	M	R	M	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
2	R	L	L	L	R	M	L	L	L	L	R	M	R	M	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
3	R	L	L	L	R	M	L	L	L	L	L	R	M	R	M	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
4	R	L	L	L	R	M	L	L	L	L	L	R	M	R	M	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
7	R	L	L	L	R	M	L	L	L	L	L	R	M	R	M	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
8	R	R	L	L	R	R	R	R	R	R	M	M	R	R	R	M	L	L	R	L	L	L	L	L	L	L	L	L	L	L	L	R	L	L	L	L	L	M	L	L	
13	R	R	L	L	R	R	R	R	R	R	M	M	R	R	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M	L	L	
15	R	R	M	L	R	M	M	L	L	L	L	R	M	R	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M	L	L	
16	R	R	M	M	L	R	M	L	L	L	L	R	M	R	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M	L	L	
21	R	M	M	L	R	M	L	L	L	L	L	R	M	R	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M	L	L	
23	R	L	L	L	R	M	L	L	L	L	L	L	R	R	M	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M	L	L	
27	R	L	L	L	R	M	L	L	L	L	L	L	R	R	M	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M	L	
30	R	L	L	L	R	M	L	L	L	L	L	L	R	R	M	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M	L	
31	R	L	L	L	R	M	L	L	L	L	L	L	R	R	M	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M	L	
33	R	L	L	L	R	M	L	L	L	L	L	L	R	R	M	L	L	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M	L	
34	H	L	L	L	R	M	L	L	L	L	L	M	R	R	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
35	H	R	M	L	R	M	L	L	M	M	L	M	R	R	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
42	H	L	L	L	R	M	L	L	M	M	L	M	R	R	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
45	R	H	L	L	R	M	L	L	L	L	L	M	R	R	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
47	R	L	L	L	R	M	L	L	L	L	L	M	R	R	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
50	R	L	L	L	R	M	L	L	L	L	L	M	R	R	M	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
59	R	R	L	L	R	L	M	R	R	L	L	M	R	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
64	R	R	L	L	R	L	M	R	R	L	L	M	R	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
68	R	R	L	L	R	L	M	R	R	L	L	M	R	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
71	L	L	L	L	R	L	M	R	R	L	L	M	R	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
72	R	L	L	L	R	L	M	R	R	L	L	M	R	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
73	R	L	L	L	R	L	M	R	R	L	L	M	R	R	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	

**Reactivity Chart 6. Protection for the Carboxyl Group (Continued)**

[illegible]

### Reactivity Chart 7. Protection for the Thiol Group

1. *S*-Benzyl Thioether
3. *S*-*p*-Methoxybenzyl Thioether
5. *S*-*p*-Nitrobenzyl Thioether
6. *S*-4-Picolyl Thioether
7. *S*-2-Picolyl *N*-Oxide Thioether
8. *S*-9-Anthrylmethyl Thioether
9. *S*-Diphenylmethyl Thioether
10. *S*-Di(*p*-methoxyphenyl)methyl Thioether
12. *S*-Triphenylmethyl Thioether
15. *S*-2,4-Dinitrophenyl Thioether
16. *S*-*t*-Butyl Thioether
19. *S*-Isobutoxymethyl Monothioacetal
20. *S*-2-Tetrahydropyranyl Monothioacetal
23. *S*-Acetamidomethyl Aminoethioacetal
25. *S*-Cyanomethyl Thioether
26. *S*-2-Nitro-1-phenylethyl Thioether
27. *S*-2,2-Bis(carboethoxy)ethyl Thioether
30. *S*-Benzoyl Derivative
36. *S*-(*N*-Ethylcarbamate)
38. *S*-Ethyl Disulfide

(See chart, pp. 733-735.)

Reactivity Chart 7. Protection for the Thiol Group

PG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	30	36	38
	A. AQUEOUS										B. BASIC										C. NUCLEOPHILIC									
	pH < 1, 100°										pH > 12, 150°										NaOMe									
	pH < 1										PhCN										Emolate									
	pH 1										(C <sub>10</sub> H <sub>8</sub> ) <sup>+</sup> Na <sup>+</sup>										RS <sup>+</sup> , N <sub>3</sub> <sup>+</sup> , SCN <sup>-</sup>									
	pH 2-4										MeSOCH <sub>2</sub> Na <sup>+</sup>										OAc <sup>-</sup> , X <sup>-</sup>									
	pH 4-6										KO <sup>-</sup> t-Bu										MeCN, pH 12									
	pH 6-8.5										LiN-1-Pr <sub>2</sub>										HCl, pH 6									
	pH 8.5-10										Py; R <sub>3</sub> N										R <sub>2</sub> L									
	pH 10-12										NaNH <sub>2</sub>										RMgX									
	pH > 12										NaH										Organozinc									
	pH > 12, 150°										PhCN										Organocopper									
	pH < 1, 100°										pH > 12, 150°										Wittig; ylide									
	pH < 1										pH > 12, 150°										H <sub>2</sub> /Raney (Ni)									
	pH 1										pH > 12, 150°										H <sub>2</sub> /Pt pH 2-4									
	pH 2-4										pH > 12, 150°										H <sub>2</sub> /Pd									
	pH 4-6										pH > 12, 150°										H <sub>2</sub> /Lindlar									
	pH 6-8.5										pH > 12, 150°										H <sub>2</sub> /Rh									
	pH 8.5-10										pH > 12, 150°										Zn/HCl									
	pH 10-12										pH > 12, 150°										Zn/HOAc									
	pH > 12										pH > 12, 150°										C <sub>6</sub> (H <sub>5</sub> ), pH 5									

PC	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	P.
	L. OXIDANTS																O. MISCELLANEOUS															
	I <sub>2</sub> PhSeX, PhSeCl Br <sub>2</sub> , Cl <sub>2</sub> MnO <sub>2</sub> /CH <sub>2</sub> Cl <sub>2</sub> NaIO <sub>4</sub> pH 5-8 SeO <sub>2</sub> pH 2-4 SeO <sub>2</sub> /Py K <sub>2</sub> Fe(CN) <sub>6</sub> , pH 8 Pb(IV), 25° Pb(IV), 80° Ti(NO <sub>3</sub> ) <sub>3</sub> 150° 250° 350° :CCl <sub>2</sub> N <sub>2</sub> CHCO <sub>2</sub> R/Cu CH <sub>2</sub> I <sub>2</sub> /Zn(Cu) R <sub>3</sub> SnH/In. Ni(CO) <sub>4</sub> CH <sub>2</sub> N <sub>2</sub> SOCl <sub>2</sub> Ac <sub>2</sub> O, 25° Ac <sub>2</sub> O, 80° DCC MeI Me <sub>3</sub> O <sup>+</sup> BF <sub>4</sub> 1. LDA 2. MeI 1. K <sub>2</sub> CO <sub>3</sub> 2. MeI RCHO RCOCl C <sup>+</sup> /olefin																															

Reactivity Chart 7. Protection for the Thiol Group (Continued)

PC	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77		
	G.																L. OXIDANTS																								
	Na/NH <sub>3</sub> Al(Hg) SnCl <sub>2</sub> /Py HSO <sub>3</sub> <sup>-</sup> ; H <sub>2</sub> S LiAlH <sub>4</sub> Li- <i>n</i> -Bu <sub>3</sub> BH (C <sub>5</sub> H <sub>11</sub> ) <sub>2</sub> BH B <sub>2</sub> H <sub>6</sub> , 0° NaBH <sub>4</sub> Zn(BH <sub>4</sub> ) <sub>2</sub> NaBH <sub>3</sub> CN pH 4-6 Li( <i>o</i> -Bu) <sub>2</sub> AlH Li( <i>o</i> -Bu) <sub>3</sub> AlH AlCl <sub>3</sub> , 80° AlCl <sub>3</sub> , 25° SnCl <sub>4</sub> ; BF <sub>3</sub> LiClO <sub>4</sub> ; MgBr <sub>2</sub> TsOH, 80° TsOH, 0° Hg(II) Ag(I) Cu(II)/Py HBr/In. HX/In. NBS/CCl <sub>4</sub> Br <sub>2</sub> CCl <sub>4</sub> /In. OsO <sub>4</sub> KMnO <sub>4</sub> , pH 7, 0° O <sub>3</sub> , -50° RCO <sub>3</sub> H, 0° RCO <sub>3</sub> H, 50° CrO <sub>3</sub> /Py CrO <sub>3</sub> , pH 1 H <sub>2</sub> O <sub>2</sub> , pH 10-12 Quinone DMSO, 100° NaOCl pH 10 aq NBS																																								

Reactivity Chart 7. Protection for the Thiol Group (Continued)

### Reactivity Chart 8. Protection for the Amino Group: Carbamates

1. Methyl Carbamate
5. 9-Fluorenylmethyl Carbamate
8. 2,2,2-Trichloroethyl Carbamate
11. 2-Trimethylsilylethyl Carbamate
16. 1,1-Dimethylpropynyl Carbamate
20. 1-Methyl-1-phenylethyl Carbamate
22. 1-Methyl-1-(4-biphenyl)ethyl Carbamate
24. 1,1-Dimethyl-2-haloethyl Carbamate
26. 1,1-Dimethyl-2-cyanoethyl Carbamate
28. *t*-Butyl Carbamate
30. Cyclobutyl Carbamate
31. 1-Methylcyclobutyl Carbamate
35. 1-Adamantyl Carbamate
37. Vinyl Carbamate
38. Allyl Carbamate
39. Cinnamyl Carbamate
44. 8-Quinolyl Carbamate
45. *N*-Hydroxypiperidinyl Carbamate
47. 4,5-Diphenyl-3-oxazolin-2-one
48. Benzyl Carbamate
53. *p*-Nitrobenzyl Carbamate
55. 3,4-Dimethoxy-6-nitrobenzyl Carbamate
58. 2,4-Dichlorobenzyl Carbamate
65. 5-Benzisoxazolylmethyl Carbamate
66. 9-Anthrylmethyl Carbamate
67. Diphenylmethyl Carbamate
71. Isonicotinyl Carbamate
72. *S*-Benzyl Carbamate
75. *N*-(*N'*-Phenylaminothiocarbonyl) Derivative

(See chart, pp. 737–739.)

Reactivity Chart 8. Protection for the Amino Group: Carbamates

[illegible]

Reactivity Chart 8. Protection for the Amino Group: Carbamates (Continued)

[illegible]



1. *N*-Formyl
2. *N*-Acetyl
3. *N*-Chloroacetyl
5. *N*-Trichloroacetyl
6. *N*-Trifluoroacetyl
7. *N*-*o*-Nitrophenylacetyl
8. *N*-*o*-Nitrophenoxycetyl
9. *N*-Acetoacetyl
12. *N*-3-Phenylpropionyl
13. *N*-3-(*p*-Hydroxyphenyl)propionyl
15. *N*-2-Methyl-2-(*o*-nitrophenoxyl)propionyl
16. *N*-2-Methyl-2-(*o*-phenylazophenoxyl)propionyl
17. *N*-4-Chlorobutyl
19. *N*-*o*-Nitrocinnamoyl
20. *N*-Picolinoyl
21. *N*-(*N'*-Acetylmethionyl)
23. *N*-Benzoyl
29. *N*-Phthaloyl
31. *N*-Dithiasuccinoyl

(See chart, pp. 741-743.)

Reactivity Chart 9. Protection for the Amino Group: Amides

[illegible]

**Reactivity Chart 9. Protection for the Amino Group: Amides (Continued)**

[illegible]

Reactivity Chart 9. Protection for the Amino Group: Amides (Continued)

PC	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	
Na/NH <sub>3</sub>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Al (Hg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
SnCl <sub>2</sub> /Py	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
H <sub>2</sub> SO <sub>5</sub> , H <sub>2</sub> S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
LiAlH <sub>4</sub>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
Li <sub>2</sub> H-Bu <sub>3</sub> BH	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
(C <sub>5</sub> H <sub>11</sub> ) <sub>2</sub> BH	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
B <sub>2</sub> H <sub>6</sub> , 0°	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
NaBH <sub>4</sub>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
Zn (BH <sub>4</sub> ) <sub>2</sub>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
NaBH <sub>3</sub> CN pH 4-6	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
Li (OEtBu) <sub>3</sub> AlH	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
AlCl <sub>3</sub> , 80°	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
AlCl <sub>3</sub> , 25°	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
SnCl <sub>4</sub> , BF <sub>3</sub>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
LiClO <sub>4</sub> , MgBr <sub>2</sub>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
TsOH, 80°	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
TsOH, 0°	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
Hg (II)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
Ag (I)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
Cu (II)/Py	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
HBr/Ln <sup>+</sup>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
HX/Ln <sup>+</sup>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
NBS/CCl <sub>4</sub>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
Br <sub>2</sub> CCl <sub>4</sub> /Ln <sup>+</sup>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
OsO <sub>4</sub>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
KMnO <sub>4</sub> , pH 7, 0°	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
O <sub>3</sub> , -50°	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R																							

### Reactivity Chart 10. Protection for the Amino Group: Special -NH Protective Groups

1. *N*-Allyl
2. *N*-Phenacyl
3. *N*-3-Acetoxypropyl
5. Quaternary Ammonium Salts
6. *N*-Methoxymethyl
8. *N*-Benzyloxymethyl
9. *N*-Pivaloyloxymethyl
12. *N*-Tetrahydropyranyl
13. *N*-2,4-Dinitrophenyl
14. *N*-Benzyl
16. *N*-*o*-Nitrobenzyl
17. *N*-Di(*p*-methoxyphenyl)methyl
18. *N*-Triphenylmethyl
19. *N*-(*p*-Methoxyphenyl)diphenylmethyl
20. *N*-Diphenyl-4-pyridylmethyl
21. *N*-2-Picolyl *N'*-Oxide
24. *N,N'*-Isopropylidene
25. *N*-Benzylidene
27. *N*-*p*-Nitrobenzylidene
28. *N*-Salicylidene
33. *N*-(5,5-Dimethyl-3-oxo-1-cyclohexenyl)
37. *N*-Nitro
39. *N*-Oxide
40. *N*-Diphenylphosphinyl
41. *N*-Dimethylthiophosphinyl
47. *N*-Benzenesulfonyl
48. *N*-*o*-Nitrobenzenesulfonyl
55. *N*-2,4,6-Trimethylbenzenesulfonyl
56. *N*-Toluenesulfonyl
57. *N*-Benzylsulfonyl
59. *N*-Trifluoromethylsulfonyl
60. *N*-Phenacylsulfonyl

(See chart, pp. 745-747.)

Reactivity Chart 10. Protection for the Amino Group: Special -NH Protective Groups

Reagent	1	2	3	5	6	8	9	12	13	14	16	17	18	19	20	21	24	25	27	28	33	37	40	41	47	48	55	56	57	59
1. <i>N</i> -Allyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
2. <i>N</i> -Phenacyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
3. <i>N</i> -3-Acetoxypropyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
5. Quaternary Ammonium Salts	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
6. <i>N</i> -Methoxymethyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
8. <i>N</i> -Benzyloxymethyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
9. <i>N</i> -Pivaloyloxymethyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
12. <i>N</i> -Tetrahydropyranyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
13. <i>N</i> -2,4-Dinitrophenyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
14. <i>N</i> -Benzyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
16. <i>N</i> - <i>o</i> -Nitrobenzyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
17. <i>N</i> -Di( <i>p</i> -methoxyphenyl)methyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
18. <i>N</i> -Triphenylmethyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
19. <i>N</i> -( <i>p</i> -Methoxyphenyl)diphenylmethyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
20. <i>N</i> -Diphenyl-4-pyridylmethyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
21. <i>N</i> -2-Picolyl <i>N'</i> -Oxide	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
24. <i>N,N'</i> -Isopropylidene	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
25. <i>N</i> -Benzylidene	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
27. <i>N</i> - <i>p</i> -Nitrobenzylidene	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
28. <i>N</i> -Salicylidene	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
33. <i>N</i> -(5,5-Dimethyl-3-oxo-1-cyclohexenyl)	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
37. <i>N</i> -Nitro	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
39. <i>N</i> -Oxide	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
40. <i>N</i> -Diphenylphosphinyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
41. <i>N</i> -Dimethylthiophosphinyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
47. <i>N</i> -Benzenesulfonyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
48. <i>N</i> - <i>o</i> -Nitrobenzenesulfonyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
55. <i>N</i> -2,4,6-Trimethylbenzenesulfonyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
56. <i>N</i> -Toluenesulfonyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
57. <i>N</i> -Benzylsulfonyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
59. <i>N</i> -Trifluoromethylsulfonyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
60. <i>N</i> -Phenacylsulfonyl	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L

Reactivity Chart 10. Protection for the Amino Group: Special – NH Protective Groups (Continued)

[illegible]